

**THE EFFECT OF PURE TITANIUM AND TITANIUM ALLOY ON
OSTEOBLAST AND CHONDROCYTE MATURATION *IN VITRO***

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THESIS**

Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
at San Antonio
in Partial Fulfillment
of the Requirements
for the Degree of
Master of Science

By
Jack Herbert Lincks, DDS

San Antonio, Texas

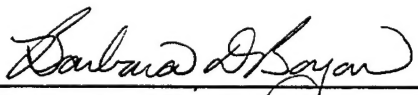
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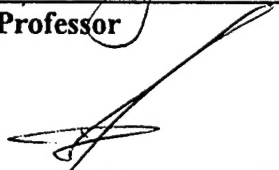
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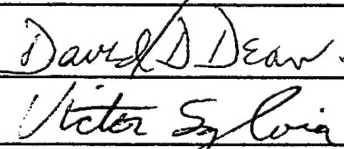
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David D. Dean



Victor S. Lewis



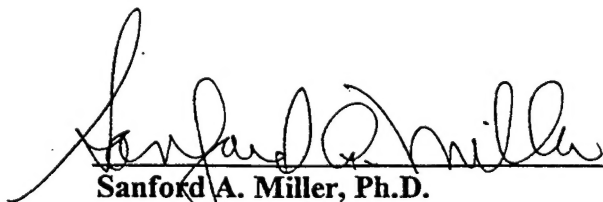
Mark L. Farnsworth



David H. Barker

Date February 12, 1999

APPROVED:



Sanford A. Miller, Ph.D.
Dean

DEDICATION

I dedicate this thesis to my best friend and wife Jennifer, whose relentless support, incredible patience, discerning vision, witty humor, and unconditional love has sustained me through this work, the residency and the other arduous times of our lives together. To Kaitlyn my wondrous daughter, Evan my jovial boy and Brian the newest addition to our family. You are pure love, the greatest of Gods blessings, and without your smiles this endeavor would have been impossible. I also wish to recognize my parents, John and Jeanne Lincks, who have always believed in me and instilled confidence in my abilities to accomplish any task. To all I am forever grateful.

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THE ROLE OF PURE TITANIUM AND TITANIUM ALLOY ON OSTEOBLAST AND CHONDROCYTE MATURATION IN VITRO

Jack Herbert Lincks, M.S.

The University of Texas Graduate School of Biomedical Sciences
at San Antonio

Supervising Professor: Barbara D. Boyan, Ph.D.

The Effect Of Implant Surface Roughness And Composition On Osteoblast Differentiation And Cytokine Production.

Implant success is dependent on the interaction of the implant with surrounding tissues. By understanding this, better devices can be developed. Substrate specific information regarding implant surface characteristics and its influence on mesenchymal cell proliferation and differentiation may be helpful in the design of implants to optimize bone growth at the interface. Based on *in vivo* and *in vitro* studies, biomaterial composition, surface energy, surface roughness, and surface topography appear to play a major role in implant success. However, in-depth analysis of cellular response to various implant surface characteristics and the ensuing local and systemic factor production remain to be performed. The first aim of this study was to evaluate the ability of two implant characteristics, chemical composition and surface roughness, to

modulate various parameters of osteoblast metabolism *in vitro*. Commercially pure titanium (Ti) and titanium-6 wt.% aluminum-4 wt.% vanadium alloy (Ti-A) disks were machined to achieve a specific roughness through either fine-polishing (S, smooth, R_a 0.2 μm) or wet sand-grinding (R, rough, R_a 3.0 μm). Profilometry and scanning electron microscopy (SEM) were used to determine surface roughness, Auger spectroscopy and FT-IR were also used to characterize the disks. MG63 osteoblast-like human osteosarcoma cells were seeded at 9300 cells/cm² and cultured to confluence on standard tissue culture polystyrene or the Ti surfaces. Cell morphology was assessed by SEM. Osteoblast cultures were examined for differences in proliferation as measured by cell number, and [³H]-thymidine incorporation; differentiation by cell and cell layer alkaline phosphatase specific activity (ALPase) and osteocalcin production; matrix synthesis by proteoglycan sulfation and % collagen production; and cytokine production by transforming growth factor- β and prostaglandin E₂ production.

Cell proliferation was reduced on all metal surfaces as compared to polystyrene control. Proliferation on Ti-R was similar to Ti-A-R which was less than that seen on smooth surfaces. Only cells grown on Ti-R exhibited a decrease in cell number. ALP was greater on the metal surfaces than on plastic and demonstrated a surface roughness dependent increase: Ti-R > Ti-A-R > Ti-S = Ti-A-S > plastic. MG63 cells grown on Ti-R produced twice as much osteocalcin as cells grown on other surfaces. Only MG63 cells grown on Ti-R had similar % collagen production to cells grown on plastic, whereas cells grown on Ti-A-R, Ti-S, and Ti-A-S had reduced production. All metal surfaces inhibited proteoglycan production as compared to control. Local factor production as measured by TGF β and PGE₂ showed a surface roughness dependent increase: Ti-R > Ti-A-R > Ti-S = Ti-A-S > plastic. Moreover, there was significantly more factor production by cells cultured on Ti-R compared to Ti-A-R.

The second aim of this study was to evaluate the ability of two implant characteristics, chemical composition and surface roughness, to modulate various parameters of chondrocyte

metabolism *in vitro*. Chondrocytes at two different stages of endochondral differentiation, the less mature resting zone chondrocyte (RC) and more mature growth zone chondrocytes (GC), isolated from the costochondral cartilage of 125 g Sprague-Dawley rats were cultured to confluence. 24 hours after confluence cellular response was measured by assaying cell number, [^3H]-thymidine incorporation for differences in proliferation; differentiation by cell and cell layer alkaline phosphatase specific activity (ALPase); proteoglycan sulfation and % collagen production for matrix production; and local factor production by transforming growth factor- β , and prostaglandin E_2 production. Both cell number and [^3H]-thymidine incorporation was reduced in GC and RC cultures on rough surfaces but not on smooth surfaces when compared to control. Cellular ALPase was less on rough surfaces compared to plastic, whereas smooth surfaces showed no difference: $\text{Ti-S} = \text{Ti-A-S} = \text{plastic} > \text{Ti-R} = \text{Ti-A-R}$. RC cell layer ALPase was also less on rough surfaces, however, GC cell layer ALPase was increased on Ti-R. % collagen production was reduced in RC cells on Ti-R while in GC cells it was increased on Ti-R. No change was seen on other surfaces for either cell line. All metal surfaces inhibited proteoglycan production as compared to control for both cells. Local factor production as measured by TGF β and PGE_2 showed a surface roughness dependent increase: $\text{Ti-R} > \text{Ti-A-R} > \text{Ti-S} = \text{Ti-A-S} > \text{plastic}$ for both cells. Moreover, there was greater factor production by cells cultured on Ti-R compared to Ti-A-R.

This study indicates that bone cell proliferation, differentiation, matrix synthesis and local factor production are affected by surface roughness and bulk composition. Osteoblast and chondrocyte maturation was enhanced for cells cultured on rougher surfaces. Enhanced differentiation of cells grown on rough vs. smooth surfaces for both Ti and Ti-A surfaces was indicated by decreased proliferation and increased ALPase and osteocalcin production. Local factor production was also enhanced on rough surfaces, supporting the contention that these cells are more differentiated. Likewise, surface composition played a role in cell differentiation and

local factor production for both osteoblasts and chondrocytes. Cells cultured on Ti-R surfaces produced more ALPase as well as TGF β than those cultured on Ti-A-R. These results imply that surface roughness and chemical composition plays a major role in the cellular response at the bone-implant interface. Moreover, differences in RC and GC response suggest chondrocytes are not only affected by surface roughness and chemical composition but their response is cell maturation dependent. Taken together, these results suggest the best design for a dental or orthopaedic implant has a pure titanium surface with rough microtopography.

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I. INTRODUCTION AND LITERATURE REVIEW

For an implant to be successful, mature bone must form in direct apposition to the implant, in a process called osseointegration (Branemark, 1985). In an effort to affect osseointegration, much emphasis has been placed on the bone-implant interface and the implant characteristics which promote bone apposition. Achieving implant osseointegration begins with an understanding of the bone wound healing process and is completed through successful bone formation. The initial interplay between the bone and implant primarily determines the success or failure of the implant. When an implant is placed in bone, trauma occurs at the site and a localized acute inflammatory reaction ensues. This initial response may result in either rejection of the implant through an immunological response or the more desired host-implant compatibility.

Researchers have intensely studied the macroscopic mechanical properties of implant materials in an effort to promote osseointegration. Currently biomaterial research has emphasized the microscopic characteristics of implant materials such as, biomaterial composition, surface energy, roughness, and microtopography (Rich and Harris, 1981, Thomas and Cook, 1985). Yet the search for a biomaterial with the desired macroscopic and microscopic properties to promote wound healing at the bone-implant interface continues. Biomaterial surface characteristics may enhance wound healing by promoting the production of extracellular serum proteins and cytokines by host tissues, as well as, recruiting undifferentiated mesenchymal cells to the wound site. These proteins and cytokines then coat the implant surface providing optimal conditions for cell attachment to the implant, and directing the proliferation and differentiation of the mesenchymal cells (Jarcho et al., 1977, Schwartz and Boyan, 1994). Once differentiated, the cells produce a rich organic matrix called osteoid, containing extracellular organelles called matrix vesicles, and

growth factors such as prostaglandin E₂ (PGE₂), and transforming growth factor beta (TGFβ). These growth factors and other chemotactic factors are released at the cell-biomaterial interface and may modulate the cellular activity in the surrounding tissue via auto- or paracrine pathways (Ziats et al., 1988). As the cells mature they begin to mineralize the matrix and promote bone formation, a process regulated by systemic hormones and locally produced cytokines (Davies et al., 1990, Schwartz et al., 1994) that ultimately results in osseointegration. In many cases where implants are used, the surrounding tissue goes through a healing process involving endochondral bone formation. Therefore, chondrocytes are one of the first cell types to see an implant and to be affected by its surface properties.

1. Titanium Alloy as an Implant Material

A wide variety of metals, polymers, and ceramics have been used in an attempt to find the ideal implant material. Titanium and titanium alloys are the most frequently used implant materials in orthopedics and dentistry because of their biocompatibility, excellent mechanical properties, and resistance to corrosion (Williams 1981, Albrektsson 1984). While pure titanium is currently the material of choice for uncoated implants in dentistry, titanium alloys, particularly the aerospace alloy Ti-6AL-4V, have become the materials of choice in orthopedics and for coated dental implants (Van Noort, 1987, Grabowsky et al., 1989). The strength requirements of surgical implants in orthopedics have virtually eliminated the use of pure titanium while titanium alloy (Ti-6AL-4V) has proven to be an excellent biocompatible material with superior mechanical properties (Bordji et al., 1996).

The biocompatibility of Ti-6AL-4V implants, however, is not without controversy. Little is known about the release of trace elements *in vivo* from dental or orthopedic implants. The potential toxicity of aluminum and vanadium ions passively released from the implant surface or from physiological wear may have negative effects on osseointegration (Thompson and Puleo 1996). *In vitro* studies examining the effects of metal ion leaching reveal localized cytotoxic

effects on mesenchymal cells affecting both the morphology and the structure of the tissue surrounding the implant; however, the extent and significance of this toxicity is not known (Michel 1984, McKay et al., 1996). Using immortalized neonatal rat calvarial osteoblasts, McKay et al., (1996) showed vanadium concentrations of 50 μM to be toxic to osteoblasts after 48 hour exposure with virtually no toxic effect from aluminum. He speculated that lower concentrations may be toxic upon chronic exposure. Using physiologically relevant, sublethal concentrations of aluminum and vanadium, Thompson and Puleo (1996), postulated that these ions suppressed rat bone marrow stromal cell differentiation since osteocalcin production and subsequent osteoid mineralization were inhibited with little effect on cell proliferation and alkaline phosphatase activity. Other studies have found that aluminum ions affected the post-translational modification of osteocalcin in ROS 17/2 cells, suggesting that extracellular levels of osteocalcin might be suppressed by metal ions (Fanti et al., 1992). This work correlates well with previous findings by others (Blumenthal and Posner, (1984), Blumenthal and Cosma (1989), and Eisenbarth et al., 1996) suggesting that Ti-6AL-4V released metal ions inhibited hydroxyapatite formation by poisoning active crystal growth sites. Ion release from titanium alloy is implicated in increasing the width and decreasing the efficiency of the amorphous bonding zone at the bone-implant interface, as compared to pure titanium (Albrektsson, 1984). Additional studies suggest that aluminum and vanadium ions may stimulate the release of inflammatory mediators, which initiate a macrophage response and stimulate fibroblast proliferation that ultimately contribute to osteolysis and implant failure (Haynes, et al., 1993, Maloney et al., 1993). If the *in vivo* response is similar to *in vitro* results, then Ti-6AL-4V implants may actually decrease osseointegration and promote implant failure.

2. Effects of Implant Composition

The chemical composition of an implant has a direct effect on the biological response of mesenchymal cells and influences their migration, attachment, and differentiation. For example, proliferation, metabolism, extracellular matrix synthesis, and differentiation are affected when osteoblasts and chondrocytes are cultured on different substrates (Schwartz et al., 1991, Hambleton et al., 1994). In addition, Sinha et al. (1994), demonstrated that implant composition affects osteoblast attachment, spreading, cytoskeletal organization, and focal contact formation. The chemical composition of an implant can also be altered following exposure to and absorption of extracellular proteins produced at the implant site. Corrosion products and ion leaching have also been shown to alter cellular response in some materials (Galante 1972, Winter 1974, Michel 1984).

Titanium-containing metals have been shown to osseointegrate better than other materials due to the formation of a 2.5 nm thick titanium oxide layer (Albrektsson et al., 1981 Williams 1981, Kesemo and Lausmaa, 1988a and 1988b, Sanford and Keller, 1991). Both pure titanium and titanium alloys develop a surface oxide layer due to the natural passivation of titanium (Rostoker and Galante, 1979, Lausmaa et al., 1986). However, differences in the crystallinity of the underlying metal as well as the segregation of alloy components within the metal itself, may cause the oxide that forms on pure titanium to be quite different from the oxide that forms on titanium alloys. Several studies have shown that subtle differences in surface composition, including titanium oxide crystallinity, can modify cell response, even when surface roughness is unchanged (Golijanin and Bernard, 1988, Golijanin et al., 1989, Nowlin et al., 1989, Arai et al., 1989, Michaels et al., 1989, Hambleton et al., 1994, Evans and Benjamin, 1987, Merrit and Brown, 1985).

3. Effects of Implant Surface Roughness

In vivo and *in vitro* studies have shown that variations in implant surface microtopography affect cellular response. Thomas and Cook (1985) found that implants with rough surfaces have greater shear strength and more direct bone apposition than those with smooth surfaces which produce more fibrous encapsulation. Further, *in vivo* studies have shown that rough surfaces induce more osteogenesis than smooth ones (Wilke et al., 1990, Buser et al., 1991, Cochran et al., 1994). *In vitro*, rough surfaces enhance bone formation while smooth surfaces promote fibrous attachment (Schroeder et al., 1981, Michaels et al., 1989, Bowers et al., 1992). Grooves and pores encourage cell orientation allowing for increased attachment, synthesis of extracellular matrix and subsequent mineralization (Albrektson et al., 1981, Brunette 1988, Groessner-Scheiber and Tuan, 1992). Increased surface area of rough surface implants may lead to greater absorption of cell attachment proteins, formation of focal adhesions, and increased production of cytokines (Kieswetter et al., 1996, Boyan et al., 1996). It has been shown that surface roughness alters phenotypic expression of osteoprogenitor cells (Schwartz and Boyan 1994). For example, on surfaces created by grit blasting or chemical etching, cells may form focal attachments which are different from those formed on machined surfaces with the same roughness. It has been speculated that focal adhesion and cell orientation alter phenotype expression of bone producing cells (Schwartz and Boyan 1994, Boyan et al., 1995).

Cultured osteoblasts and chondrocytes are affected by surface roughness (Martin et al., 1995, Schwartz et al., 1996, Boyan et al., 1997). These cells exhibit different rates of cell proliferation, and levels of differentiation and matrix production when grown on commercially pure titanium disks of varying roughness, indicating cellular activity is affected by substrate microtopography. When compared to cells grown on smoother surfaces, cells grown on rougher surfaces produced more matrix, expressed more alkaline phosphatase, and released higher levels of prostaglandin E₂ (PGE₂), and transforming growth factor β (TGF- β) (Martin et al., 1995,

Schwartz et al., 1996, Kieswetter et al., 1996). The overall similarity in response between MG63 osteoblast-like cells and resting zone chondrocytes (RC) suggests that response to surface roughness is dependent on cell maturation. Current research suggests that rough surfaces may promote osseointegration by encouraging differentiation of mesenchymal cells to osteoblasts (Boyan et al., 1996).

4. Markers of Cell Differentiation

The ability of osteogenic cells to synthesize an appropriate matrix and then subsequently mineralize it is essential for bone formation. For our studies we have used two indices which are recognized as important markers of osteoblastic differentiation: alkaline phosphatase activity and osteocalcin production.

Alkaline Phosphatase. Alkaline phosphatase has been implicated in playing a critical role in mineralization and bone formation (Ali 1980) and is recognized as an important index of chondrocyte and osteoblast cell differentiation. Alkaline phosphatase is enriched in matrix vesicles and is considered their primary marker enzyme. Matrix vesicle maturation is indicated by increased alkaline phosphatase content in the tissues adjacent to osteogenic materials (Boyan et al., 1993). Alkaline phosphatase is believed to promote the hydrolysis of phosphate-containing organic substrates releasing phosphate which is essential for matrix calcification (Cyborg et al., 1982).

Osteocalcin. Osteocalcin, the major noncollagenous protein of bone, accounts for 10 to 20% of the noncollagenous protein in bone and is associated with the mineral phase. Osteocalcin is thought to play a role in bone matrix formation and remodeling (Bonewald et al., 1992) and is considered to be a terminal marker of osteoblast differentiation, since calcification directly correlates with increased osteocalcin production (Aranow et al., 1990).

5. Markers of Matrix Synthesis

The initial matrix, or osteoid, produced by differentiated mesenchymal cells is rich in organic material. Approximately 90% of the matrix is collagen which forms a network or scaffold for mineralization. The extracellular matrix also contains numerous noncollagenous proteins which include proteoglycans, α -carboxyglutamate containing proteins such as osteocalcin, acid glycoproteins, sialoproteins, serum proteins, and cytokines such as PGE₂, BMP-2, and TGF- β (Ali 1980, Reddi et al., 1978, Reddi 1994)

Collagen Production. Chondrocytes have been shown to express mRNA for Type I and Type II collagen in culture (Finer 1985). *In vitro* studies have shown that Type I collagen is expressed by differentiating osteoblasts (Mundy 1995). Collagen synthesis and matrix production appear to be influenced by implant surface properties. Using embryonic chick calvarial cells Groessner-Schreiber and Tuan, 1992, demonstrated enhanced collagen matrix synthesis and matrix calcification on rough titanium and titanium-alloy surfaces. Similar results have been observed with MG63 osteoblast-like cells and growth zone (GC) chondrocytes (Martin et al., 1995, Schwartz et al., 1996). In general pre-osteoblastic cells grown on smoother surfaces demonstrated decreased collagen synthesis while cells grown on rough surfaces showed a significant increase in percent collagen production. These data suggest that cells cultured on rougher surfaces express a more differentiated phenotype. Surface chemical composition has also been shown to alter chondrocyte collagen synthesis *in vitro*. Using various sputter-coated surfaces, Hambleton et al., 1994, found decreased collagen production by cells cultured on zirconium, aluminum oxide, and calcium phosphate ceramic. Cells cultured on sputtered coated titanium surfaces produced collagen at levels similar to those seen on the plastic controls. These results suggest that chondrocytes are also affected by subtle differences in surface chemical composition.

Proteoglycans. Proteoglycans are macromolecules with glycosaminoglycan chains bound to a protein core. Their main function is biomechanical, as structural elements within the matrix, however, they may also regulate mineralization (Reddi et al., 1978, Sela et al., 1992). Within the extracellular matrix, proteoglycans occupy the interstitial spaces between the collagen framework and mesenchymal cells. Proteoglycan-collagen complexes have been postulated to act as nucleators of collagen fiber formation (Reddi 1994). Proteoglycan aggregates are inhibitors of mineralization and must be removed prior to mineralization. It has been hypothesized that mature matrix vesicles release metalloproteinases which degrade proteoglycans and allow extracellular matrix calcification (Dean et al., 1992). These proteases have been isolated from matrix vesicles produced by chondrocytes and osteoblasts (Dean et al., 1994). Sulfated proteoglycans are a marker of well-differentiated, mature articular chondrocytes, but the relevance of this marker to growth plate chondrocytes is less clear (Roughley et al., 1987). As chondrocytes mature along the endochondral pathway production of proteoglycan is reduced as is sulfation of existing proteoglycan (Buckwater et al., 1987).

6. Local Factor Regulation of Bone Metabolism

Local autocrine and paracrine factors are essential for the development and maintenance of mature bone (Raisz and Fall 1990, Ingram et al., 1994, Kieswetter et al., 1996). Undifferentiated mesenchymal cells migrate to the implant surface and attach, proliferate and differentiate in response to systemic and local factors. This differentiation can favor the chondrogenic phenotype in sites of low O_2 tension and the osteoblastic phenotype in sites of high O_2 tension, whereas, micromotion may lead to fibrocartilage development (Boyan et al., 1996). During cell proliferation and differentiation, extracellular proteins and growth regulatory factors are expressed and stored in the forming bone matrix. The matrix is the primary source of osteogenic growth regulatory factors which stimulate supplementary osteoblast differentiation via

autocrine and paracrine mechanisms (Mundy 1995). Several researchers have characterized the local growth factors found in bone matrix and shown that TGF- β 1 is the most abundant (Seyedin et al., 1986, 1987). Other growth factors found in bone matrix include insulin-like growth factors I and II, acidic and basic fibroblastic growth factor, platelet-derived growth factor, bone morphogenic proteins and transforming growth factor TGF- β 2 (Mundy 1995). Two local factors produced by cultured chondrocytes and osteoblasts that are significant in both wound healing and bone production are TGF- β and PGE₂ (Joyce et al., 1990, Schwartz et al., 1992, Bonewald et al., 1992, Kieswetter et al., 1996). Previous studies in our laboratory have shown that material surface characteristics alter the type and amount of cytokines produced by cells at the bone-implant interface. The hypothesis that bone cells grown on rougher surfaces are more differentiated than those grown on smoother surfaces is supported by enhanced production of local factors.

Transforming Growth Factor β Members of the transforming growth factor β superfamily play a major role in bone and cartilage morphogenesis (Joyce et al., 1990). TGF- β is important in the initial proliferation, differentiation, and matrix production of precursor cells into osteoblasts (Dworetz et al., 1990). TGF- β regulates osteoblast matrix vesicle production and stimulates the enzymes associated with calcification (Bonewald et al., 1992). TGF- β also enhances the osteoinductive properties of BMP's (Bentz et al., 1991) and interacts with 1,25(OH)₂D₃ to regulate osteoblast differentiation and matrix production (Ingram et al., 1994, Iimura et al., 1994). Kieswetter et al., (1996) demonstrated that titanium surface roughness increases the production of TGF- β and suggested that this increase may be due to the cells having acquired a more differentiated cellular phenotype.

Prostaglandin E₂ PGE₂ appears to affect bone formation and resorption. At low concentrations, PGE₂ stimulates DNA synthesis in mesenchymal cells, enhancing replication and eventual maturation into osteoblasts. It also increases osteoblast collagen synthesis. At high

concentrations PGE₂ inhibits osteoblast collagen synthesis as well as overall osteoblast activity (Raisz and Fall, 1990). In chondrocyte cultures, it has been shown that PGE₂ production is regulated by 1,25-(OH)₂D₃ and the effect appears to be cell-maturation dependent (Schwartz et al, 1992c). Production of PGE₂ by MG63 osteoblast-like cells is enhanced when cultured on rough surfaces. Furthermore, the addition of 1,25-(OH)₂D₃ to the culture media stimulated an even greater production of PGE₂ on rough surfaces while no effect was seen on smooth surfaces (Boyan et al., 1998). This synergistic effect appears to be partially mediated by locally produced PGE₂ since blocking PGE₂ with indomethacin abolished the effects of surface roughness on all parameters (Boyan et al., 1998). It has been speculated that PGE₂ may stimulate production of cytokines associated with cellular proliferation and differentiation (McCarthy et al., 1991).

7. Purpose and Aims of the Current Study

The success or failure of an implant is dependent in part on its surface characteristics. We have previously showed that when MG63 osteoblast-like osteosarcoma cells and rat costochondral cartilage cells (RCs and GCs) are cultured on pure titanium discs with average surface roughness values (Ra's) varying from <0.1 µm (smooth) to 3-4 µm (rough) to >6 µm (very rough), there are distinct differences in cell response (Martin et al., 1995, Schwartz et al., 1996). For these studies, the smooth surfaces were obtained by electropolishing following chemical etching; rough surfaces were obtained by coarse grit blasting; and very rough surfaces were achieved via Ti plasma spray. The results showed that as surface roughness increased, expression of markers suggestive of a more differentiated osteoblastic and chondrocyte phenotype increased. The optimal surface appeared to be one with an Ra values around 4 µm: cell proliferation was reduced but not blocked and phenotypic differentiation was enhanced. In contrast, cells on smooth surfaces had higher proliferation rates while alkaline phosphatase activity and osteocalcin production were reduced, indicating a less differentiated phenotype. The

purpose of the current study was to determine what role, if any does chemical composition and microtopography play in regulating the phenotype of mineralizing cells. To do this we examined the cellular response of MG63 osteoblast-like cells and costochondral chondrocytes to machined surfaces by culturing these cells on disks of varying roughness that were prepared from pure titanium and Ti-6Al-4V alloy. The results of the present study were compared to those of previous work using commercially pure titanium disks that had been acid etched or grit blasted to obtain similar Ra values (Martin et al., 1995, Schwartz et al., 1996).

The first specific aim of this study was to determine if implant chemical composition (pure titanium and titanium alloy) and surface roughness (smooth $R_a < 0.5 \mu\text{m}$ and rough $R_a > 3.0 \mu\text{m}$) alter the cellular response of MG63 osteoblast-like cells. Many of the previous studies have used transformed cell lines to examine the effect of implant characteristics on host tissues. These transformed cell lines may have lost certain typical traits or characteristics which could conceivably alter their response *in vitro*. Therefore, the second specific aim of this study used primary cultures of rat costochondral cartilage cells at two stages of maturation to determine if implant chemical composition (pure titanium and titanium alloy) and surface roughness (smooth $R_a < 0.5 \mu\text{m}$ and rough $R_a > 3.0 \mu\text{m}$) alter cellular response. These primary cultures allow for study of endochondral maturation at two distinct stages (RCs are less mature than GCs with respect to endochondral differentiation). Therefore, it is possible to examine how cells at specific states of maturation might respond to the material surface. This investigation tested the hypothesis that chemical composition and surface roughness modulate the response of MG63 cells, as well as, resting zone (RC) and growth zone (GC) chondrocytes to implants.

II. MATERIALS AND METHODS

1. Titanium Disk Preparation

Titanium disks (14.75 mm in diameter and 0.80 mm thick) were fabricated from sheets of medical grade, commercially pure Titanium (cpTi: medical grade 2, ASTM F67, "unalloyed Ti for medical applications") and Titanium-6 wt.% Aluminum-4 wt.% Vanadium alloy (Ti-6Al-4V) obtained from Timet (O'Fallon, MO 63366). Chemical composition was provided by the supplier and not verified prior to surface preparation. Each sheet was sectioned into 1 ft x 1 ft plates for ease of handling and to ensure a consistent finish. The disks were either polished or ground to acquire the desired surface finishes. Polishing to create the smooth disks was performed by lapping with 18T grit (oil based 500-600 grit aluminum oxide) followed by polishing with 4.0 paper (1200 grit aluminum oxide) by French Grinding Service, Inc. (Houston TX). The rough surface disks were prepared by wet sanding using a Carborundum brand zirconium oxide/aluminum oxide resin-bonded to a cloth belt by Metal Samples, Inc. (Mumford AL). Oversight of the subcontractors and verification of the characteristics of the disks was performed by Southwest Research Institute, (San Antonio TX).

Following punching out from the sheets, degreasing of the disks was accomplished by ultrasonic cleaning in acetone for one hour, followed by a second cleaning in Jet-A jet fuel (grade AL-24487-F; Diamond Shamrock, San Antonio, TX) in an ultrasonic cleaner for one hour. The disks were then washed with Versa Clean (Fisher Scientific, Pittsburgh, PA) four times with two distilled water rinses between each wash. After the final wash, the disks were rinsed with 70% ethanol, dried in a vacuum chamber, and packaged in small plastic bags. Prior to use each disk was washed with ethanol three times and rinsed with double distilled water three times. The disks were individually wrapped in gauze to prevent surface damage, and then sterilized by autoclaving

at 270°F for 20 minutes at 18 lb./in². For all experiments, cells were cultured on disks placed in 24-well plates (Corning, NY). Controls consisted of cells cultured directly on the polystyrene (tissue culture plastic) surface of the 24-well plate.

2. Titanium Disk Surface Analysis

Representative disks from each group were subjected to surface analysis as follows:

Surface Profilometry. Surface roughness was quantified using a diamond stylus contact profilometer (Taylor-Hobson Surtronic 3 profilometer, Leicester UK). Average Surface roughness (R_a) was evaluated at ten different locations on each plate to obtain an accurate determination of the roughness of the surface. For the smooth surfaces measurements were made in all directions, whereas on rough surfaces measurements were taken perpendicular to the machine markings. Following the punching operation, four disks from each 1 X 1 ft sheet were randomly sampled and roughness measured as before to assure quality control and confirm the average roughness values (R_a). The targeted R_a value for the smooth disks was $<0.5 \mu\text{m}$. While that for the rough disks was $>3.0 \mu\text{m}$. The mean and standard deviations for the R_a values for each group were statistically analyzed for significance. R_a values were not determined for the polystyrene culture dishes.

Scanning Electron Microscopy. The surface microtopography of the disks was examined by scanning electron microscopy. Particular attention was also given to consistency and examining the disks for the presence of surface defects. This was accomplished using a Amray 1645 cold field emission scanning electron microscope, (Amray, Bedford, MA). Two samples from each group were examined at two magnifications: 100X and 500X.

Auger Electron Spectroscopy. Analysis of the titanium oxide layer was performed by Auger electron spectroscopy (Perkin Elmer PHI 595, Perkin Elmer, Physical Electronics Division, Eden Prairie, MN). Spectra were obtained from two randomly selected disks from only the

smooth groups (Ti and Ti-A), since rough surfaces yield unreliable Auger spectra. Rough disks were not examined to avoid artifacts associated with rough morphologies. Further, the thickness and composition of the surface oxides on the rough and smooth disks for each material would be expected to be identical since all disks were machined and cleaned using the same protocol. To characterize the chemical nature of the titanium oxide layer and determine the near-subsurface chemical profiles, atomic monolayers were argon-milled away between Auger scans at a controlled, quantified rate. The latter was established using special standards fabricated by sputter-coating pure Ti onto glass substrates, these were then fractured and inspected by SEM to accurately define the Ti film thickness. An approximate overall ion milling rate was defined in terms of time required to remove the Ti coating causing the disappearance of the Ti Auger signal. The spectra were obtained at regular sputtering intervals at a sputtering rate of 400 Å/min. (Hambleton et al., 1994)

Fourier transform infrared spectroscopy (FTIR). Spectra from four randomly chosen disks from the smooth groups (two from the smooth Ti group and two from the smooth Ti-A group) were analyzed to determine if any organic residue remained on the samples. FTIR spectroscopy was performed using a Nicolet Magna FTIR in reflection mode. Spectra were collected using 32 scan summations at a resolution of 16 cm⁻¹. Rough samples cannot be analyzed by FTIR because artifactual measurements are obtained with rough morphologies.

3. Cell Model and Culture

MG63 human osteoblast-like cells and rat costochondral cartilage chondrocytes were used for these experiments.

Osteoblasts. The MG-63 osteoblast-like cell line was originally obtained from an osteosarcoma in the femur of a fourteen-year-old male in 1975. It is a particularly desirable cell line to use for studies of osteoblasts because it maintains its osteoblast-like phenotype while

proliferating through many cell passages. MG63 cells exhibit many of the characteristics of typical osteoblast-like cells but are believed to represent a less differentiated stage of osteoblastic maturation (Davies et al., 1990). These cells have been well-characterized and similar to osteoblasts, demonstrate increased production of alkaline phosphatase activity and osteocalcin synthesis in response to treatment with $1,25-(\text{OH})_2\text{D}_3$ (Franceschi et al., 1985, 1987). MG-63 cells were obtained from the American Type Culture Collection (Rockville, MD).

For the proposed experiments, MG-63 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (diluted from a stock solution containing 5000 U/ml penicillin, 5000 U/ml streptomycin; GIBCO, Grand Island, NY) at 37°C in an atmosphere of 5% CO_2 in air at 100% humidity. Cells were plated at a density of 9300 cells/cm². The media were changed every 48 hours until the cells reached confluence on polystyrene. When cells reached visual confluence on tissue culture polystyrene, cultures on all of the other surfaces were treated as confluent since the opacity of the Ti disks make it impossible to assess confluence. All harvests were performed as described below for each of the assays.

Chondrocytes. Resting zone (RC) and growth zone (GC) chondrocytes were derived from rat costochondral cartilage as described by Boyan et al. (1988). Briefly, ribcages were removed from 125g male Sprague-Dawley rats and placed in Dulbecco's modified Eagle's Medium (DMEM). The resting zone and adjacent growth zone cartilage were dissected and the intervening tissue removed to limit cross-contamination of cell zones. The chondrocytes were then released from the tissue by digestion with trypsin and collagenase prior to incubation. Cells were separated from the digest and then seeded into T25 flasks containing DMEM, 10% fetal bovine serum (FBS), 50 mg/ml ascorbic acid, and 1% penicillin and streptomycin. The cultures were maintained in an atmosphere of 5% CO_2 in air and 100% humidity at 37 °C. Cells were plated at a density of 10,000 cells/cm² for RC cells and 25,000 cells/cm² for GC cells. The media

were changed and fed every 48 hours until the cells reached confluence. At that time, the cells were passaged for further expansion of number or plated for the experiments on the Ti disks or tissue culture polystyrene. All experiments were performed with fourth passage culture cells. Fourth passage cells were used since prior studies have shown retention of phenotype and differential responsiveness to vitamin D₃ metabolites at this passage (Boyan et al., 1988; Schwartz and Boyan, 1988; Schwartz et al., 1988). Confluence on the Ti disks was assessed as described for the osteoblasts. All harvests were performed as described below for each of the assays.

4. Determination of Cell Morphology

To determine cell morphology alterations secondary to surface characteristics, the cultures were examined by scanning electron microscopy. At harvest, the culture media were removed and the samples rinsed three times with phosphate-buffered saline (PBS) and fixed with 1% OsO₄ in 0.10 M PBS for 15-30 minutes. After fixation, the disks were rinsed with PBS, sequentially incubated for 30-45 minutes each in 50, 75, 90, and 100% *ter*-butyl alcohol and vacuum-dried. A thin layer of gold-palladium was sputter-coated onto the samples prior to examination in a JEOL 6400 FEC cold field emission scanning microscope (JEOL USA, Inc. Peabody, MA).

5. Determination of Cell Proliferation

Cell proliferation was assessed by determining cell number and [³H]-thymidine incorporation.

Cell Number. At harvest, cell cultures were washed twice with DMEM and then released from the surface by the addition of 0.25% trypsin in Hank's balanced salt solution (HBSS) containing 1 mM ethylenediamine tetraacetic acid (EDTA) for ten minutes at 37°C. The reaction was terminated by the addition of DMEM containing 10% FBS. A second trypsinization was performed in order to assure that any remaining cells had been removed from the surface. Both

trypsinized cell suspensions were combined and centrifuged at 3000 rpm for 10 minutes. The supernatant was decanted and the cell pellets were re-suspended in 200 μ l filtered normal saline (NS) and transferred into counting vials containing 9.8 ml NS. Cell number was determined using a Coulter Counter (Hialeah, FL). Cells harvested in this manner exhibited > 95% viability based on trypan blue by exclusion.

[³H]-Thymidine Incorporation. DNA synthesis was estimated by measuring [³H]-thymidine incorporation as previously described by Schwartz et al. (1989). Cells were cultured in 24 well plates on Ti disks or the plastic surface and cultured until cells on the plastic surface reached visual confluence. Four hours prior to harvest, the media were changed and fresh media containing 50 μ l [³H]-thymidine (New England Nuclear, Boston MA), had 1 μ Ci/ml added to the cultures. At harvest, the cell layers were washed twice with cold PBS, 3 times with 5% trichloroacetic acid (TCA), and then treated with ice cold saturated TCA for 30 minutes. The samples were air dried and then dissolved in 0.3 ml 1 % sodium dodecyl sulfate (SDS) at 4°C overnight and the radioactivity measured in a Beckman LS 61000C liquid scintillation counter.

6. Determination of Cell Differentiation

Cell differentiation was assessed by measuring both cell and cell layer alkaline phosphatase specific activity. In MG63 cells, osteocalcin production was also measured to assess changes in cell differentiation.

Alkaline phosphatase specific activity of isolated cells and cell layers. At harvest, either cell layers (described below), or isolated cells (described above) were prepared and their protein content determined using commercially available kits (Micro/Macro BCA, Pierce Chemical Co., Rockford, IL). Alkaline phosphatase activity was assessed by measuring the release of p-nitrophenol from p-nitrophenylphosphate at pH 10.2 as previously described (Bretaudiere and Spillman, 1984) and the specific activity calculated.

Cell layers were prepared following the method of Hale et al., (1986). At harvest, culture media were decanted and the cell layers washed twice with PBS before removal with a cell scraper. After centrifugation, the cell layer pellets were washed once more with PBS and suspended by vortexing in 500µl 0.05% Triton-X-100. Enzyme assays were performed on the Triton X-100 lysates. Before assaying, pellets were further disrupted by freeze/thawing three times. Enzyme assays were performed on both cell and cell layer lysates.

Osteocalcin Production. The production of osteocalcin were measured by use of a commercially available radioimmunoassay kit (Human Osteocalcin RIA Kit, Biomedical Technologies, Stoughton, MA). Samples were concentrated five fold by lyophilization in a speed-vacuum. Prior to assay, samples were reconstituted in 100µl Tris-saline buffer. For assay, 50µl of sample was added to 100µl human osteocalcin antiserum, 100µl [125 I]-human osteocalcin, and 200µl Tris-saline buffer and then incubated overnight at room temperature on an orbital platform shaker at approximately 80 rpm. Goat anti-rabbit antibody and polyethylene glycol (100µl each) were added to each tube the following morning. After vortexing, the samples were placed on an orbital shaker for 2 hours at room temperature. Next, 0.07 ml of Tris-saline buffer was added to each sample. Following the addition of Tris-saline buffer the solution was vortexed and centrifuged at 10,000 rpm at 4°C for 15 minutes. The supernatant was decanted and the pellet placed in scintillation cocktail. Counts were determined using a Beckman LS 61000C liquid scintillation counter. Osteocalcin concentration in the samples was determined by the normalized percentage bound over unbound (%B/BO) to a standard curve for each standard and sample.

7. Determination of Matrix Production

Matrix production was assessed by measuring [3 H]-proline incorporation into collagen and non-collagen protein and [35 S]-sulfate incorporation into proteoglycans by the cultures.

Collagen and Noncollagen Protein Synthesis. This assay is based on a method previously described by Peterkofsky and Diegelmann (1971) where collagen production is assessed by measuring the incorporation of [^3H]-proline into collagenase-digestible protein (CDP) and noncollagenase-digestible protein (NCP). Percent collagen production was determined by comparing CDP production with total CDP + NCP production (i.e., $[\text{CDP}/(\text{CDP} + \text{NCP})] \times 100$). The protein content of each fraction was determined by miniaturization of the method of Lowry et al., (1951). This assay does not take into account any degradation that may have occurred during the incubation or during sample preparation.

At confluence, media were replaced with 500 μl /well of DMEM containing 10% FBS, 1% antibiotics, 50 $\mu\text{g}/\text{ml}$ β -aminopropionitrile (Sigma, St. Louis, MO), and 10 $\mu\text{Ci}/\text{ml}$ of L[G^3H]-proline (New England Nuclear, Boston, MA). After 24 hours, media were collected and cell layers (cells and matrix) obtained by scraping and re-suspending in two 0.2 ml portions of 0.2N NaOH. Proteins were precipitated from both the collected media and cell layers by addition of 0.1 ml 100% TCA containing 1% tannic acid, washed three times with 0.5 ml 10% TCA containing 1% tannic acid, and then twice with ice-cold acetone. The final pellets from the cell layers were dissolved in 500 μl 0.05M NaOH. Digestion of the cell layer pellet was performed using highly purified collagenase (125 units/100 μl reaction solution) (Calbiochem, San Diego, CA), as previously described by Martin et al., (1995). The protein content of each sample was determined by the use of commercially available kits (Micro/Macro BCA, Pierce Chemical Co., Rockford, IL).

Proteoglycan synthesis. Proteoglycan synthesis was assessed by [^{35}S]-sulfate incorporation as described by O'Keefe et al., (1988). Previously, we found that the amount of radiolabeled proteoglycan secreted into the media by MG-63 cells, RCs and GCs was less than 15% of the total radiolabeled proteoglycan produced. Because more than 85% of the radiolabeled proteoglycan was in the cell layer, we examined the incorporation of [^{35}S]-sulfate in

the cell layer only (Martin et al., 1995, Schwartz et al., 1996). Four hours prior to harvest, [^{35}S]-sulfate was added to the media to a final concentration of 9 $\mu\text{Ci/ml}$. At harvest, the media were removed and the wells washed once with 500 μl PBS. The cell layer was collected in two 0.25 ml portions of 0.25M NaOH. The protein content was determined by use of commercially available kits (Micro/Macro BCA, Pierce Chemical Co., Rockford, IL). To measure [^{35}S]-sulfate incorporation, the total volume of the sample was adjusted to 0.7 ml by the addition of 0.15M NaCl. The sample was dialyzed in a 12,000-14,000 molecular weight cut-off dialysis membrane against buffer containing 0.15M NaCl, 20 mM Na_2SO_4 , and 20 mM Na_2PO_4 , pH 7.4, at 4°C. The dialysis solution was changed daily until the radioactivity in the dialysate reached background levels. The amount of [^{35}S]-sulfate incorporated was determined by liquid scintillation spectroscopy and was calculated as dpm/mg cell layer protein.

8. Determination of Local Factor Production

Transforming Growth Factor β (TGF- β) Production. The level of total TGF- β in the conditioned media was determined using a commercially available ELISA assay kit (Promega Corp., Madison, WI). Immediately prior to assay, 10 μl of sample was diluted with 90 ml of DMEM (1:10). Next, 50 μl of the 1:10 diluted media was further diluted by adding 200 μl of distilled PBS. The media were then acidified by the addition of 5 μl 1M HCl for fifteen minutes followed by neutralization with 5 μl 1M NaOH for 5 minutes. The acidified samples were placed in microtiter plates coated with monoclonal antibody to TGF- β for ninety minutes. The unbound proteins were removed and the wells incubated with polyclonal rabbit anti-TGF- β antibody for two hours, washed, and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody for an additional two hours. This was then followed by wash and color development steps. Color development was terminated by the addition of 1M phosphoric acid. Absorbance measurements were conducted at 450 nm using a BioRad Model 2550 Enzyme

Immunoassay Reader (Hercules, CA). Sample concentrations were determined by use of a standard curve generated under the same conditions with rhTGF- β 1. The amount of TGF- β 1 in the cell layer was not determined because of difficulties associated with quantitatively extracting this cytokine from the matrix.

Prostaglandin E_2 (PGE_2) Production. The level of PGE_2 in the media was assessed using a commercially available competitive binding radioimmunoassay kit (NEN Research Products, Boston, MA). Unlabeled PGE_2 in the sample was incubated overnight with radiolabeled PGE_2 and unlabeled anti- PGE_2 antibody. Precipitation with polyethylene glycol separated the antigen-antibody complexes from free antigen. Sample PGE_2 concentrations were determined by correlating the percentage bound over unbound counts to a standard curve (Schwartz et al., 1992b, 1992c).

9. Statistical Management of Data

Each data point represents the mean \pm standard error of the mean for six samples per group. Prior studies using these cell culture models have shown that $n=6$ is sufficient to detect statistically significant differences between groups. Data were first analyzed by two-way analysis of variance (ANOVA). When statistically significant differences were detected the Student's t-test for multiple comparisons using Bonferroni's correction was performed. Statistical significance was determined by comparing each data point to the plastic control. Differences were considered significant at $P<0.05$. Observations were validated by performing replicate experiments a minimum of two times.

III. RESULTS

1. Surface Characteristics

Disk characterization revealed the following:

Surface Profilometry: Based on profilometry (Table 1) the R_a values for the smooth disks used in these studies were similar, 0.22 μm for pure titanium and 0.23 μm for titanium alloy. The mean R_a values for the rough disks were 4.24 μm for pure titanium and 3.44 μm for titanium alloy. There was no significant differences in relative roughness between Ti-R and Ti-A-R surfaces. Both rough surfaces were significantly rougher than both smooth surfaces. The roughness of rough surface disks was 15 - 20 fold greater than smooth surface disks. The surface roughness values increased in the following order: Ti-S ($R_a = 0.22$); Ti-A-S ($R_a = 0.23$); Ti-A-R ($R_a = 3.44$); with Ti-R ($R_a = 4.24$) being the roughest.

Scanning Electron Microscopy: When the Ti-S and Ti-A-S disks were examined by scanning electron microscopy, the surfaces were found to be very similar (Figure 1A and 1C). Morphologically, the disks had small pits (2 μm in diameter) and randomly oriented scratches from the polishing operation, which were only evident at high magnification. The Ti-R and Ti-A-R disks also had a similar appearance (Figure 1B and 1D) and contained parallel, longitudinal grooves with cracks and fissures, numerous pits, and sharp serrated edges from the grinding operation. Parallel grooves of varying heights were prominent; in addition, the distance between the grooves varied. On both rough surfaces, regions of irregular, small ($<0.1\mu\text{m}$) and large sheets ($>10\mu\text{m}$) with sharp edges could be found. Additionally, the Ti-A-R surface contained areas with pits that were 10-20 μm in diameter.

Table 1.

Average surface roughness values for the Ti and Ti-alloy disks used in the present study.

| Surface | R _a value in μm |
|---------|---------------------------------------|
| Ti-R | 4.24 ± 0.13 |
| Ti-S | $0.22 \pm 0.00^{\#}$ |
| Ti-A-R | 3.44 ± 0.12 |
| Ti-A-S | $0.23 \pm 0.00^{\#}$ |

Ti and Ti alloy (Ti-A) disks were prepared with either a smooth (S) or rough (R) surface as described in the Materials and Methods. The average roughness (R_a) of the disks was determined by diamond stylus contact profilometry. Data shown in the table represents the mean \pm SEM for four disks in each group; each disk was measured in four areas. $\#p < 0.05$, smooth vs. rough surface.

Figure 1.

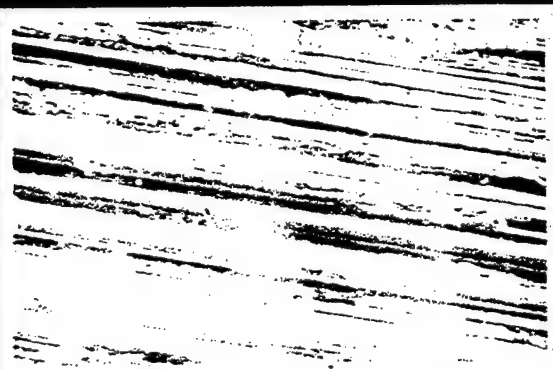
Scanning electron micrographs of the different disk surfaces used in this study. *Panel A*: Ti-S; *Panel B*: Ti-A-S; *Panel C*: Ti-R; *Panel D*: Ti-A-R. Bar = 200 μ m. Original magnification: 1000X.



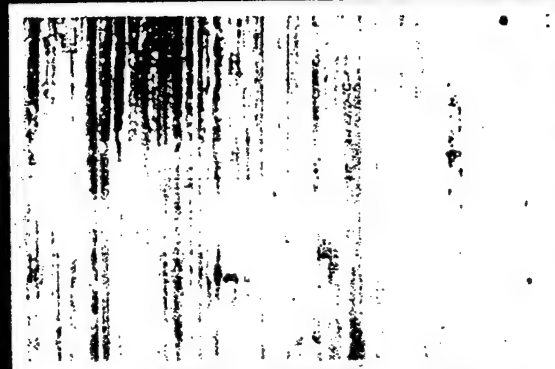
15KV X200 20μm



15KV X200 20μm



15KV X200 20μm



15KV X200 20μm

Auger Electron Spectroscopy: Before sputtering, carbon (C), nitrogen (N), titanium (Ti), and oxygen (O) were present on both smooth surfaces (Ti-S and Ti-A-S) as determined by Auger electron spectroscopy. C is always present at a material surface as a species absorbed from the atmosphere. In the alloyed surface Al was also found. After 10 seconds of sputtering at a rate of 400 Å/min the C signal was nearly gone at a depth of 67 Å in both Ti and Ti-A disks. In addition, Ti, O, and Al were present on the alloy. Twenty seconds of sputtering through the oxide layer, to a depth of 134 Å, produced a continuously decreasing O signal and an increasing Ti signal. One minute of sputtering resulted in a strong Ti signal and a diminished O signal. V was not detected due to its low concentration.

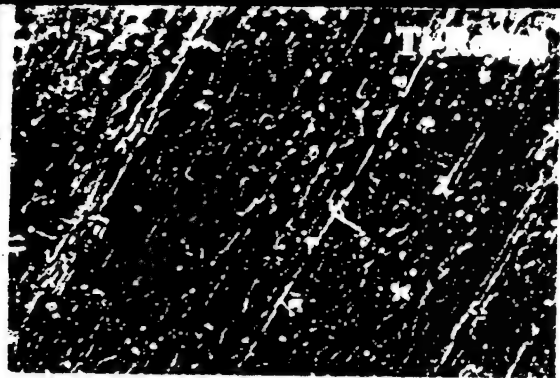
Fourier Transform Infrared Spectroscopy: FTIR analysis confirmed that no organic matter was left on the surface of either the Ti-S or Ti-A-S disks.

2. Cell Morphology

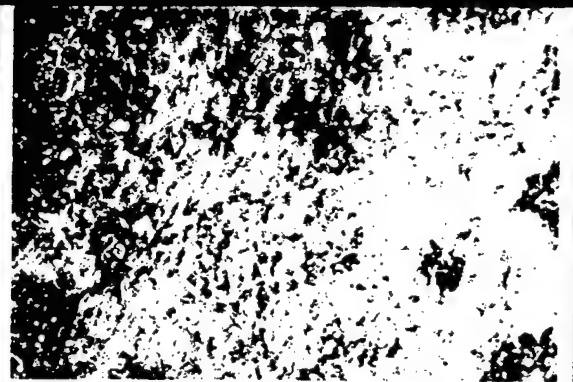
The appearance of the cells varied with surface roughness and chemical composition of the disks. Cells grown on the Ti-S surface were spread out across the surface and grew as a monolayer, but this monolayer was not continuous. The cells had a dendritic appearance with extensions that were up to 10 µm in length and had a ruffled membrane on their surface (Figures 2C and 2D). Cells cultured on Ti-R (Figures 2A and 2B) and Ti-A-S (Figures 3C and 3D) disks grew as a continuous, thin monolayer across the surface. On the Ti-R surface all cracks and fissures were covered by a monolayer of cells (Figures 2A and 2B). Cultures grown on the Ti-A-R surface induced the cells to grow as a multilayer (Figures 3A and 3B) with many cells producing extensions that covered distances of up to 10 µm. In addition, the cells grew over the sharp edges and were oriented along the parallel cracks and grooves.

Figure 2.

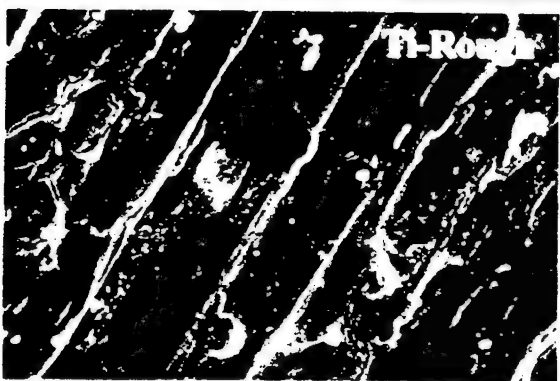
Scanning electron micrographs of MG63 osteoblast-like cells cultured on smooth and rough Ti surfaces. *Panel A*: Ti-R, magnification: 100x, bar = 10 μm ; *Panel B*: Ti-R, magnification: 500x, bar = 100 μm ; *Panel C*: Ti-S, magnification: 100x, bar = 10 μm ; *Panel D*: Ti-S, magnification: 500x, bar = 100 μm .



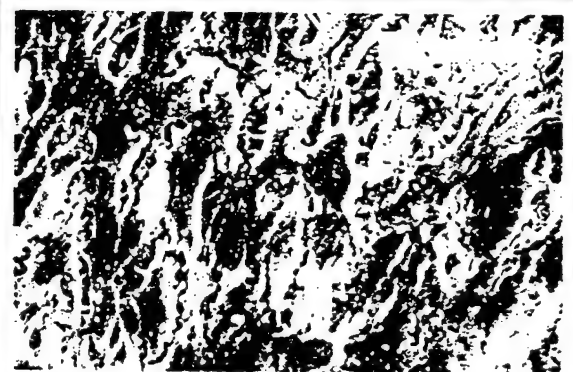
0162 15KV X100 100μm



0166 15KV X100 100μm



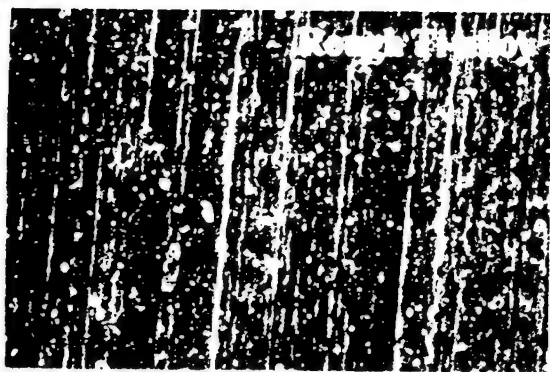
0164 15KV X500 10μm



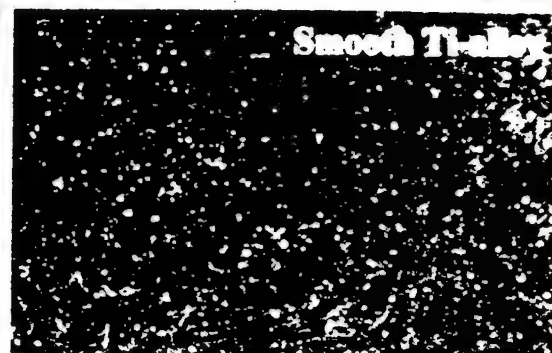
0169 15KV X500 10μm

Figure 3.

Scanning electron micrographs of MG63 osteoblast-like cells cultured on smooth and rough Ti-A-surfaces. *Panel A*: Ti-A-R, magnification: 100x, bar = 10 μm ; *Panel B*: Ti-A-R, magnification: 500x, bar = 100 μm ; *Panel C*: Ti-A-S, magnification: 100x, bar = 10 μm ; *Panel D*: Ti-A-S, magnification: 500x, bar = 100 μm .



0170 15KV X100 100μm



0303 15KV X100 100μm



0173 15KV X500 10μm



0177 15KV X500 10μm

3. Biochemical Analysis - Specific Aim #1

The objective of Aim # 1 was to examine the effect of titanium surface roughness and chemical composition on the maturation of MG63 osteoblast-like cells.

A. Cell Proliferation

Cell Number: Cell number was sensitive to surface roughness and chemical composition (Figure 4). Twenty-four hours after confluence was achieved, the number of cells on the pure titanium and the titanium alloy surfaces was similar to that found on the plastic control, except for those cultures on the Ti-R disks, which contained significantly fewer cells. Compared to plastic, cell number was reduced by 36% on Ti-R. In addition, the cell number of the Ti-R cultures was significantly less than that of the Ti-A-R cultures. The number of cells on the Ti-S surface appeared to be less than that on the control surfaces, however this reduction of cell number by 20%, was not statistically significant.

[³H]-Thymidine Incorporation: Cell proliferation as measured by [³H]-thymidine incorporation was less on all of the metal surfaces (49%) when compared to that of the plastic control (Figure 5). In addition, for the pure Ti surfaces, cell proliferation showed a surface roughness-dependent decrease, with a greater reduction seen in cultures on Ti-R (48%) than Ti-S (19%). Cell proliferation on Ti-A-S was similar to that on Ti-R and Ti-A-R which was significantly less than that seen on Ti-S.

B. Cell Differentiation

Alkaline Phosphatase Specific Activity: There was a surface roughness-dependent increase in cell layer alkaline phosphatase specific activity on all metal surfaces (Figure 6). Cell layers from cultures on all different surfaces contained significantly more alkaline phosphatase specific activity than on the plastic control (1.6 fold to 2.2 fold). Activity on the rough surfaces was consistently greater than on smoother surfaces. Cell layer alkaline phosphatase specific

Figure 4.

Number of MG63 osteoblast-like cells released by two trypsinizations of the Ti disks 24 hours after they had reached confluence on the plastic. Values are the mean \pm SEM of six cultures. * $P < 0.05$, Ti disk vs. plastic; # $P < 0.05$, Ti-A-R vs. Ti-R. Data are from one of two replicate experiments.

Effect of Disk Surface on Cell Number

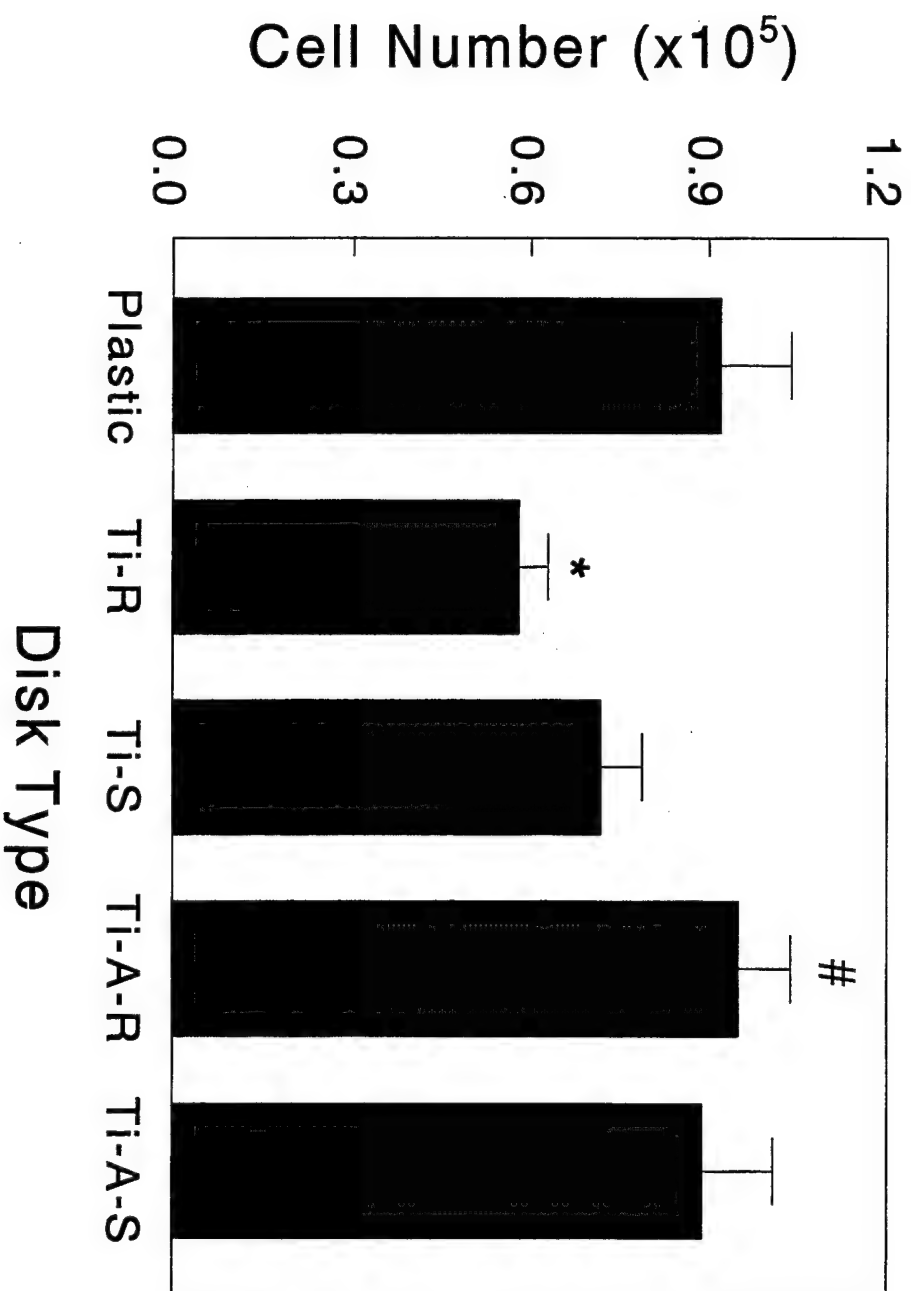


Figure 5.

[³H]-Thymidine incorporation by MG63 osteoblast-like cells during culture on plastic or Ti disks. When the cells reached confluence on plastic, the media were changed and culture continued for another 24 hours. Four hours prior to harvest, [³H]-thymidine was added and incorporation into TCA insoluble cell precipitates measured. Values are the mean \pm SEM of six cultures. *P< 0.05, Ti disk vs. plastic; #P<0.05, Ti-S vs. Ti-R. Data are from one of two replicate experiments.

Effect of Disk Surface on [³H]-Thymidine Incorporation

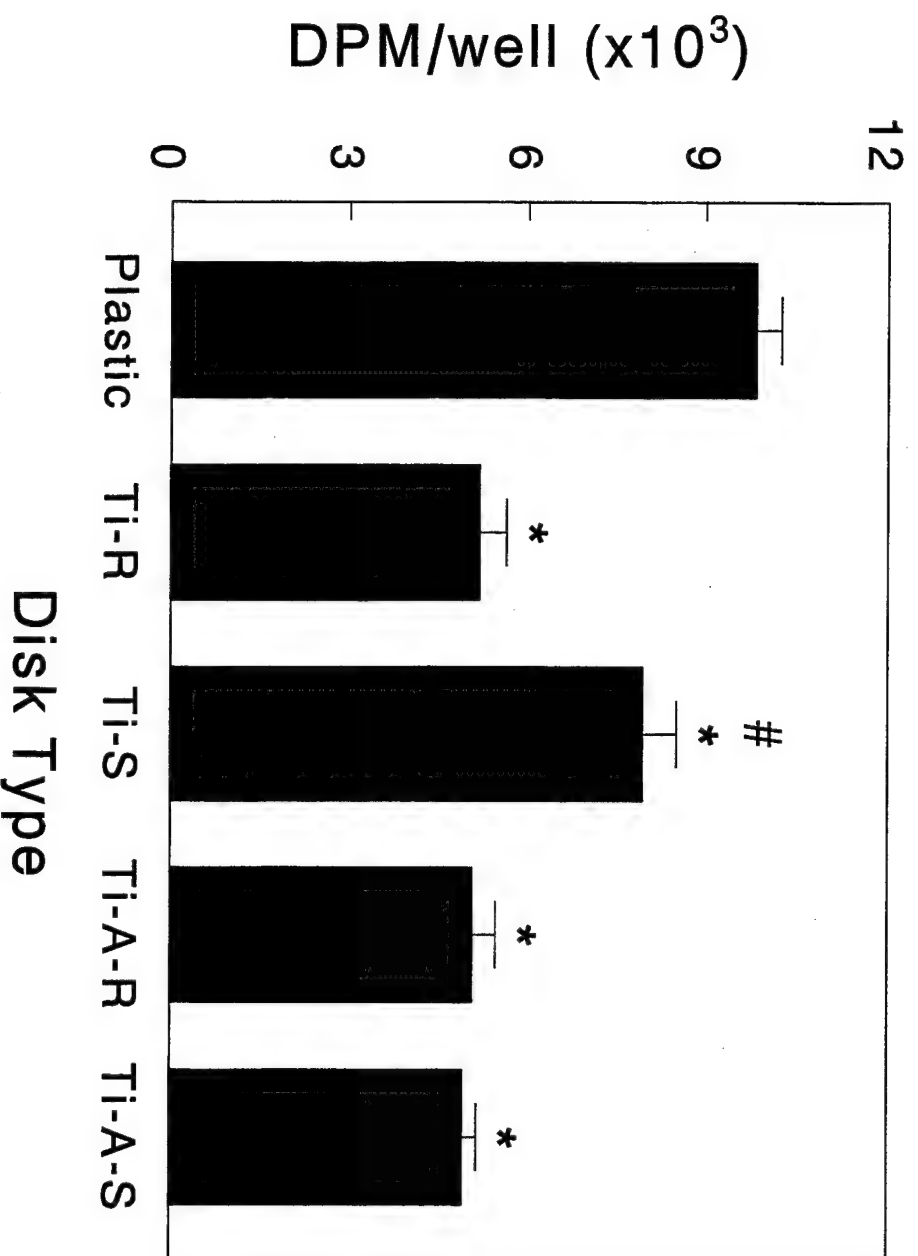
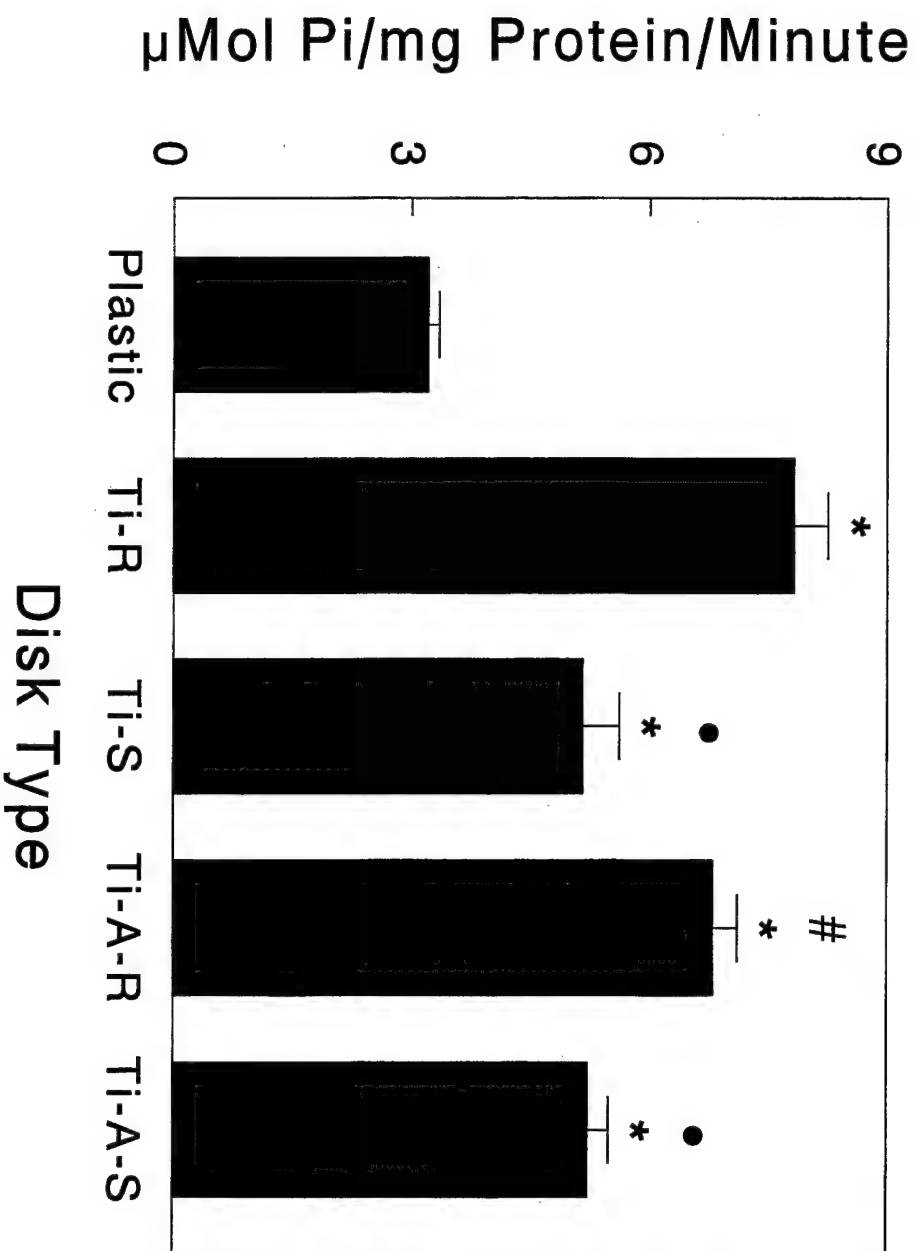


Figure 6.

Alkaline phosphatase specific activity of cell layers produced by MG63 osteoblast-like cells during culture on Ti disks. After cells had reached confluence on plastic, cultures were continued for an additional 24 hours and then harvested by scraping. Enzyme activity was measured in the cell layer lysate. Values are the mean \pm SEM of six cultures.

*P< 0.05, titanium vs plastic; #P<0.05, Ti-A-R vs Ti-R; ●P<0.05, smooth vs rough surface. Data are from one of two replicate experiments.

Effect of Disk Surface on Cell Layer ALPase Activity



activity on the Ti-R surface was 1.5 fold greater than on Ti-S. On Ti-A-R, alkaline phosphatase was 1.3 fold greater than Ti-A-S surfaces. Activity on Ti-R was 20% greater than on Ti-A-R.

Alkaline phosphatase specific activity of isolated cells was also significantly increased on all metal surfaces compared to control (Figure 7). Cells grown on Ti-R disks contained similar enzyme activity as Ti-A-R and significantly more enzyme specific activity than those on Ti-S. Cells grown on Ti-R surfaces showed a 1.8 fold increase in enzyme activity over plastic control and a 1.4 fold increase as compared to smooth surface disks. The increased enzyme activities on the smooth surface disks were 1.3 fold compared to plastic.

Osteocalcin Production: Cells cultured on Ti-R surface produced almost twice as much osteocalcin as those grown on the other surfaces (Figure 8). This 1.9 fold increase in osteocalcin production was significant for cultures grown on Ti-R disks as compared to plastic control. In contrast, cultures grown on Ti-S, Ti-A-R, or Ti-A-S surfaces produced osteocalcin levels similar to the cells grown on the plastic control.

C. Matrix Synthesis

Collagen Synthesis: Collagen synthesis was also affected by surface roughness and composition (Figure 9). While collagen synthesis was unaffected in cells grown on the Ti-R disks, cells grown on Ti-A-R, Ti-S, and Ti-A-S produced significantly less collagen (14-30% less) compared to the plastic control cultures. The percent collagen production by the cells was significantly decreased on rough Ti-A-R surface (15%) compared to Ti-R surface. Moreover, cells grown on the Ti-S surface showed a greater decrease in collagen synthesis (31%) compared to Ti-R and cells cultured on Ti-A-S produced 17% less collagen than cells grown on Ti-A-R. The collagen synthesis of cells from plastic and Ti-R surface were similar.

Proteoglycan Synthesis: Compared to control cultures, [^{35}S] sulfate incorporation was significantly reduced on all metal surfaces by 35-48% (Figure 10). MG63 cells grown on all metal surfaces exhibited comparable levels of [^{35}S] sulfate incorporation. No significant difference in

Figure 7.

Alkaline phosphatase specific activity of trypsinized MG63 osteoblast-like cells after culture on Ti disks. After cells had reached confluence on plastic, cultures were continued for an additional 24 hours and then harvested by trypsinization. Enzyme activity was measured in lysates of the cells. Values are the mean \pm SEM of six cultures. *P< 0.05, titanium vs plastic; #P<0.05, Ti-A-R vs Ti-R; ●P<0.05, smooth vs. rough surface. Data are from one of two replicate experiments.

Effect of Disk Surface on Cell ALPase Activity

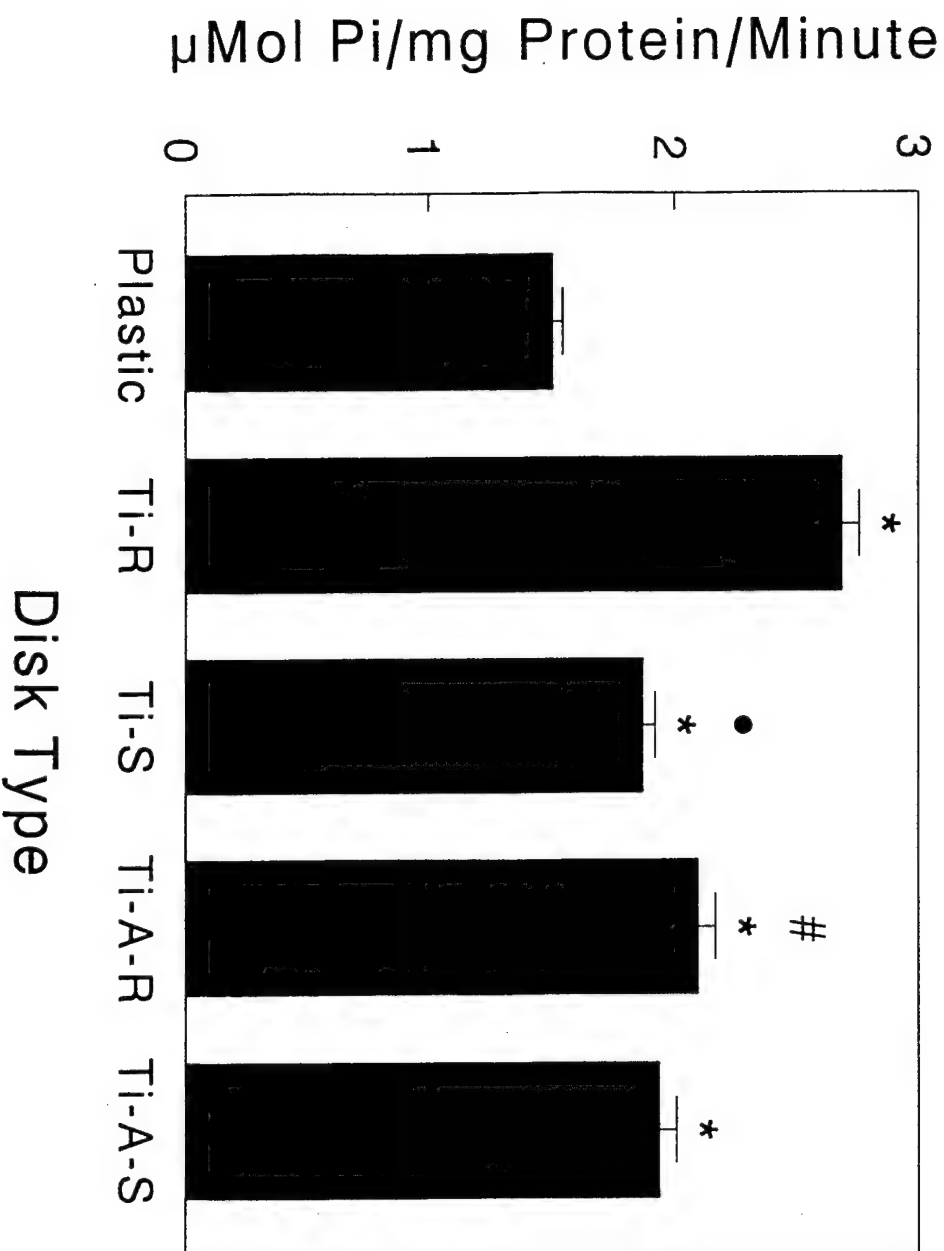


Figure 8.

Osteocalcin production by MG63 osteoblast-like cells during culture on Ti disks. After cells reached confluence on plastic, the media were changed and the culture continued for an additional 24 hours. At harvest, the media were collected, and osteocalcin content measured by RIA. Values are the mean \pm SEM of six cultures. * $P < 0.05$, titanium vs. plastic. Data are from one of two replicate experiments.

Effect of Disk Surface on Osteocalcin Production

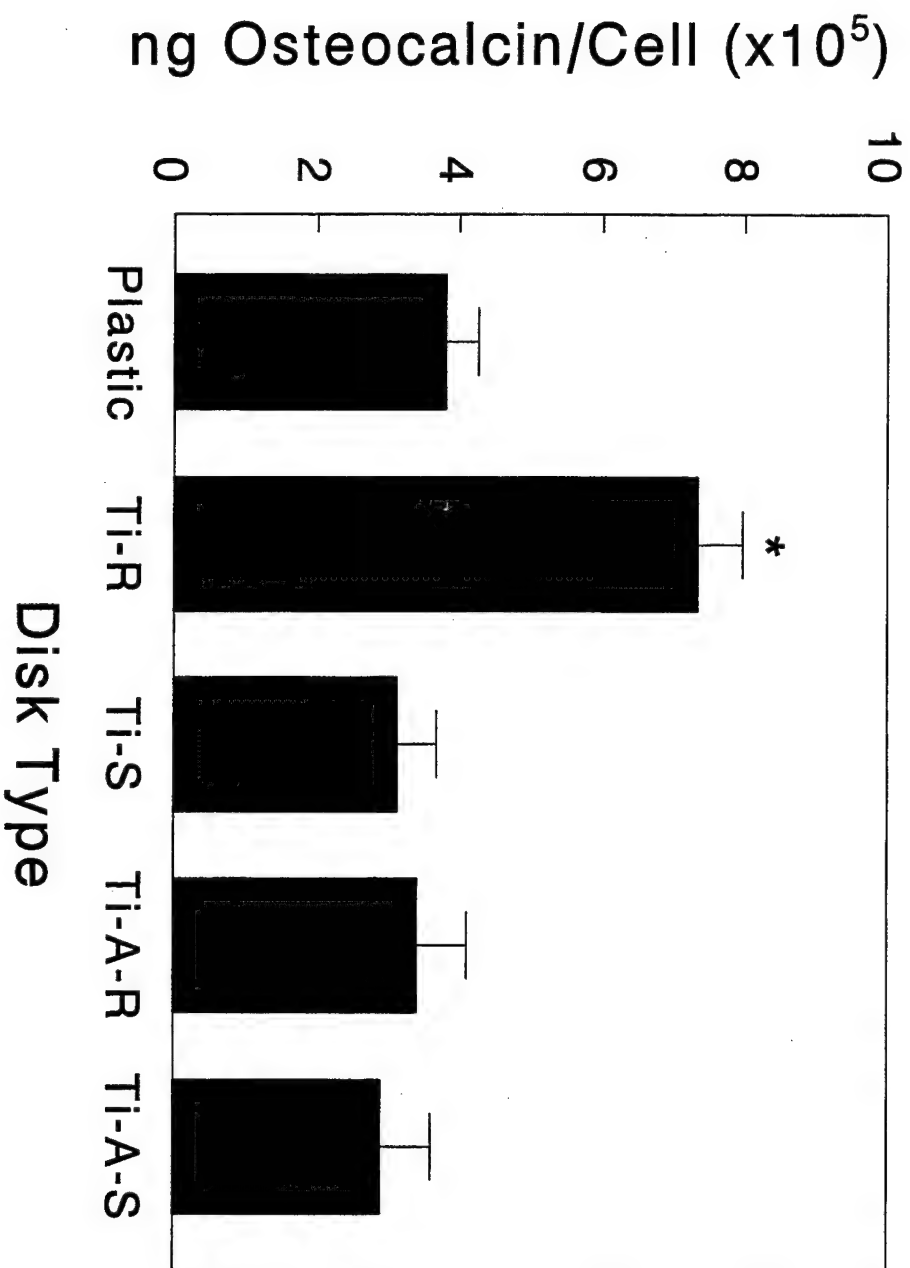


Figure 9.

Percent collagen production by MG63 osteoblast-like cells during culture on Ti disks. Values were derived from CDP and NCP production and are the mean \pm SEM of six cultures. *P< 0.05, titanium vs. plastic; #P<0.05, Ti-A-R vs Ti-R; ●P<0.05, smooth vs rough surface. Data are from one of two replicate experiments.

Effect of Disk Surface on Collagen Production

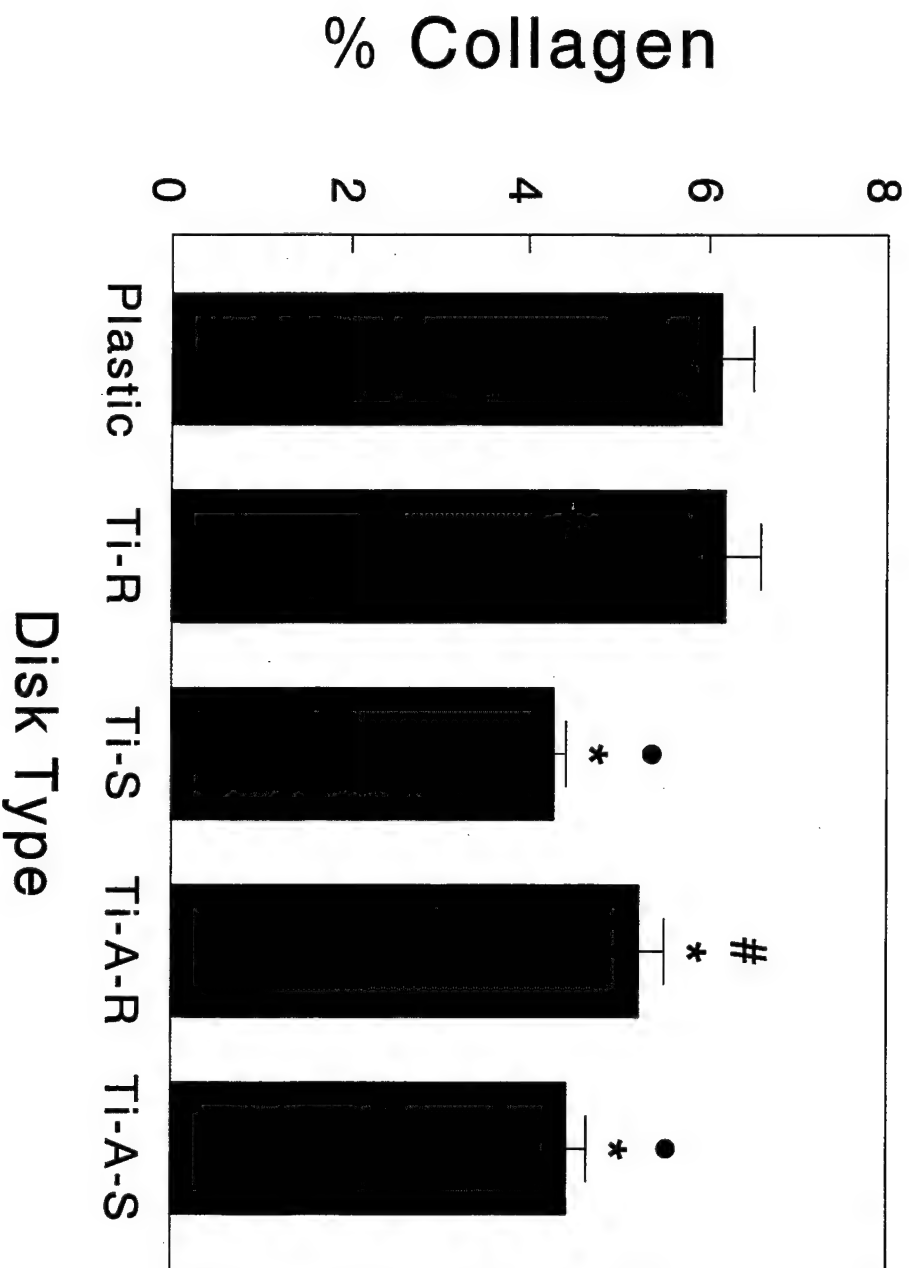
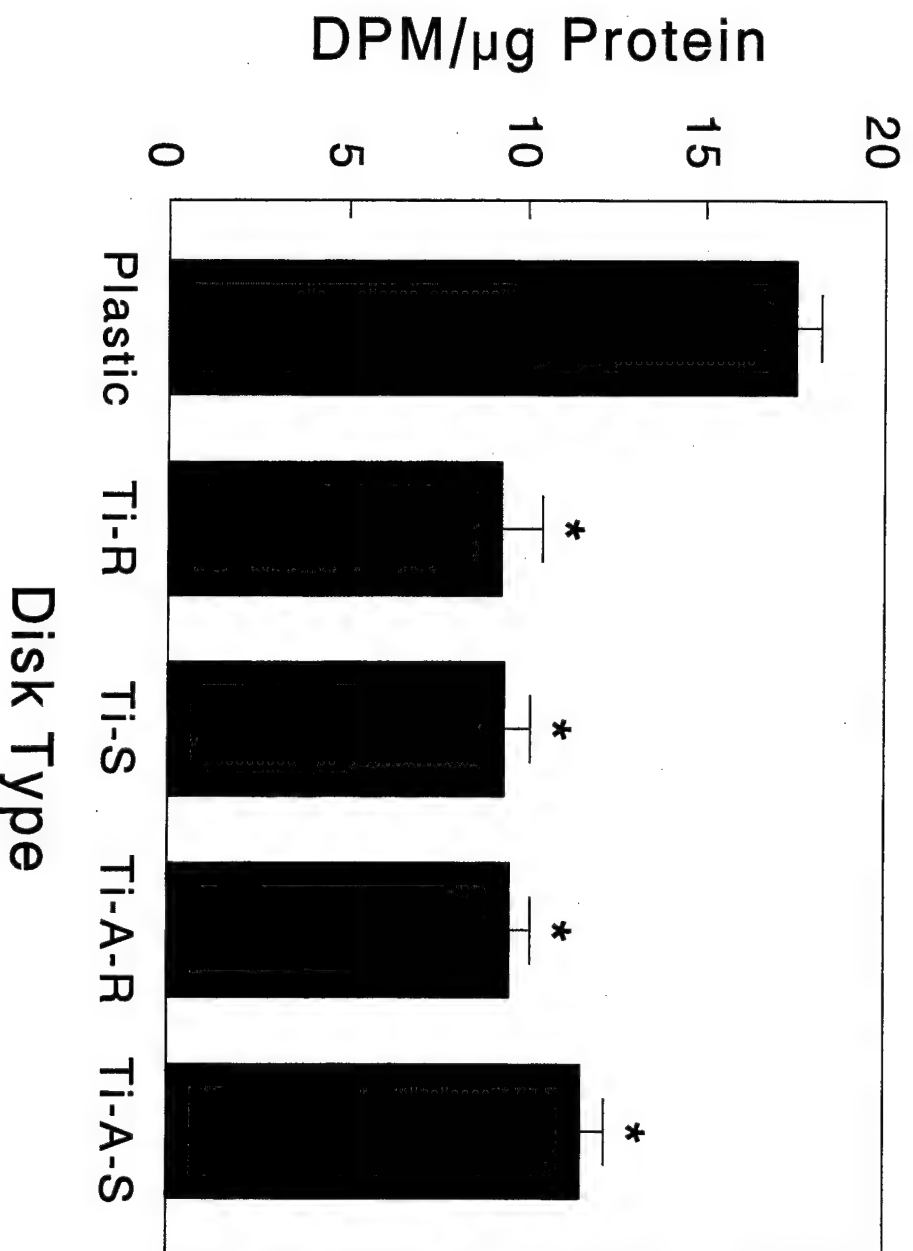


Figure 10.

[³⁵S]-Sulfate incorporation by MG63 osteoblast-like cells during culture on Ti disks. When the cells reached confluence on plastic, the media were changed and culture continued for another 24 hours. Four hours prior to harvest, [³⁵S]-sulfate was added and incorporation into the cell layer measured. Values are the mean \pm SEM of six cultures.

*P< 0.05, titanium vs plastic. Data are from one of two replicate experiments.

Effect of Disk Surface on [³⁵S]-Sulfate Incorporation



the [^{35}S]sulfate incorporation between the different surface roughness and compositions was observed.

D. Local Factor Production

TGF β Production: TGF β production by the cultures was increased with increasing surface roughness (Figure 11). Growth factor production was significantly greater on the rougher surfaces than the smoother surfaces and plastic. The TGF β levels were increased by 1.7 fold on the Ti-A-R and 2.7 fold on the Ti-R surfaces. TGF β production of cells grown on the Ti-R surface was 1.6 fold greater as compared to the cultures grown on the Ti-A-R surface and 2.1 fold compared to Ti-S. Cells grown on smooth surfaces showed no difference in TGF- β production as compared to control. Moreover, TGF- β production demonstrated a surface composition dependent increase with cells cultured on Ti-R producing significantly greater amounts of TGF- β than cells cultured on Ti-A-R surfaces.

PGE $_2$ Production: The production of PGE $_2$ also increased with surface roughness (Figure 12). PGE $_2$ release into the medium was significantly greater on Ti-R when compared to plastic (3.9 fold) and to Ti-S surface (2.0 fold). Cells cultured on Ti-A-R surfaces produced 2.9 fold more PGE $_2$ than those on the smoother Ti-A-S surfaces. In addition, cells cultured on Ti-R produced greater relative amounts of PGE $_2$ than cells cultured on Ti-A-R surfaces but this was not statistically significant. Cells grown on smooth surfaces showed no significant difference in PGE $_2$ production compared to control.

Figure 11.

Transforming growth factor β (TGF β) production by MG63 osteoblast-like cells during culture on Ti disks. After the cells reached confluence on plastic, the media were changed and the culture continued for an additional 24 hours. At harvest, the media were collected, and TGF β content measured by ELISA. Values are the mean \pm SEM of six cultures. *P< 0.05, titanium vs. plastic; #P<0.05, Ti-A-R vs Ti-R; ●P<0.05, smooth vs. rough surface. Data are from one of two replicate experiments.

Effect of Disk Surface on TGF β Production

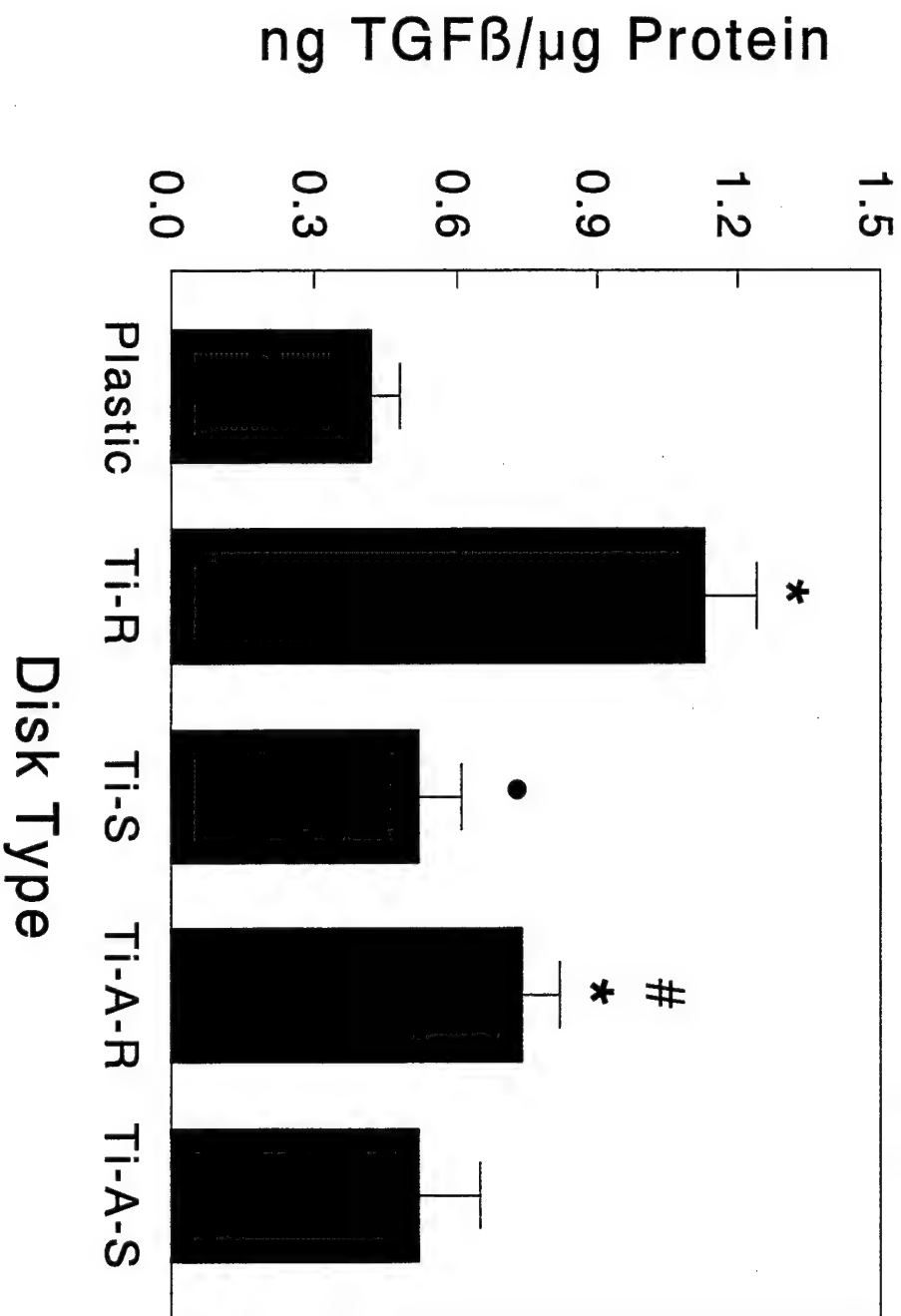
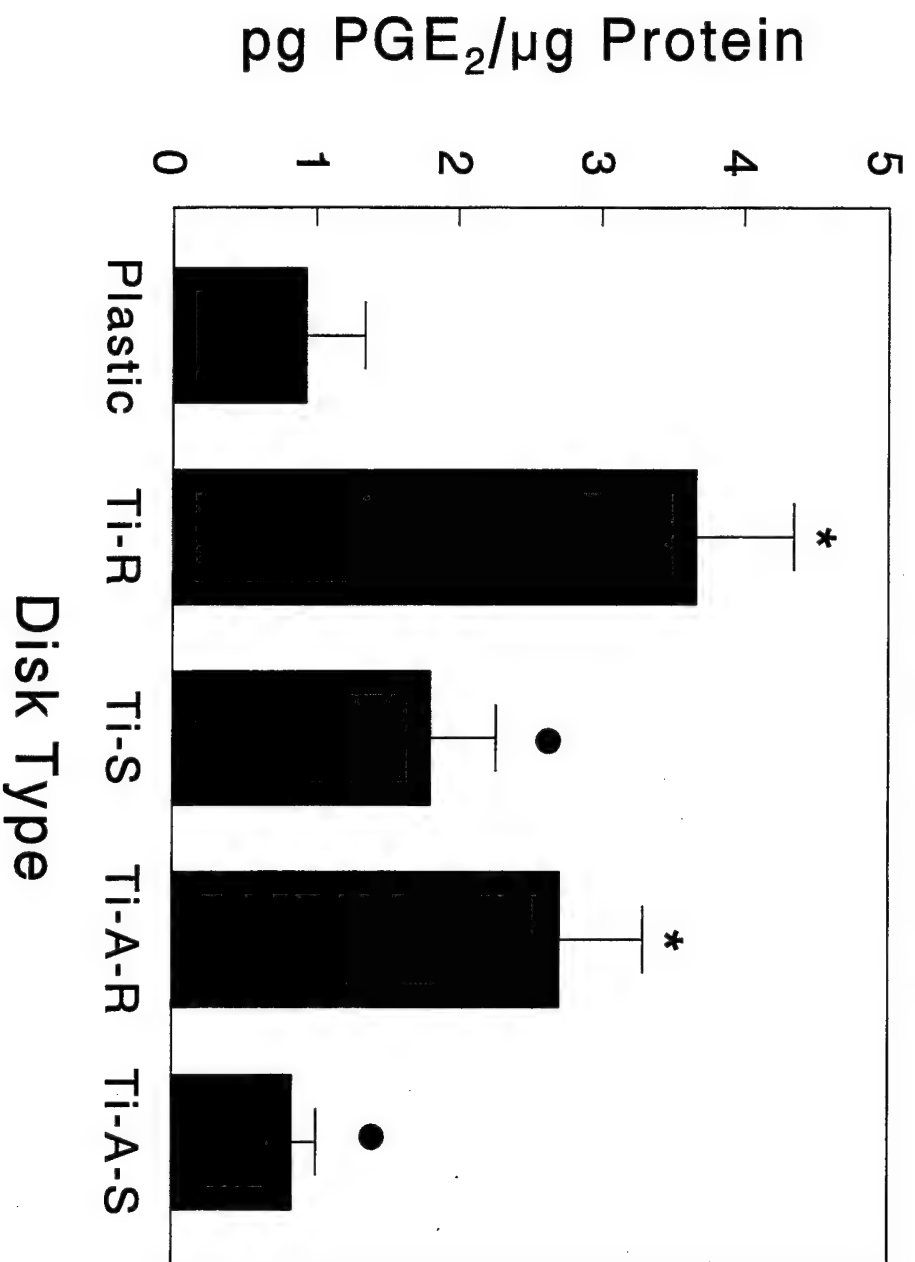


Figure 12.

Prostaglandin E₂ (PGE₂) production by MG63 osteoblast-like cells during culture on Ti disks. After cells reached confluence on plastic, the media were changed and the culture continued for an additional 24 hours. At harvest, the media were collected, and PGE₂ content measured by RIA. Values are the mean \pm SEM of six cultures. *P< 0.05, titanium vs. plastic; #P<0.05, Ti-A-R vs Ti-R; ●P<0.05, smooth vs rough surface. Data are from one of two replicate experiments.

Effect of Disk Surface on PGE₂ Production



4. Biochemical Analysis - Specific Aim #2

The objective of Aim # 2 was to examine the effect of titanium surface roughness and chemical composition on the maturation of chondrocytes.

A. Cell Proliferation

Cell Number: Surface roughness and chemical composition had an effect on the cell number of both RC and GC cell types (Figure 13). Both RCs and GCs demonstrated a surface roughness-dependent decrease in cell number that was significant for cultures on Ti-R and Ti-A-R surfaces (53% and 43%) when compared with plastic control. Further, both RC and GC cultures on Ti-R disks had significantly lower cell numbers than those on Ti-S (1.5 fold and 2.1 fold). Although cell number on the two smooth Ti surfaces, Ti-S and Ti-A-S, appeared to be less than on the control, the differences were not significant. Moreover, GCs appeared to respond to the chemical composition of the rough surfaces since significantly fewer cells were found in cultures grown on Ti-R disks than those grown on Ti-A-R disks (26%).

[³H]-Thymidine Incorporation: An inverse relationship between [³H]-thymidine incorporation and surface roughness was observed for both RCs and GCs (Figure 14). [³H]-thymidine incorporation for RCs and GCs was significantly less on rougher surfaces, Ti-R (40% and 47%) and Ti-A-R (28% and 35%), as compared to plastic control. In contrast, no differences in cell proliferation were observed for cells cultured on smooth surfaces, Ti-S and Ti-A-S, as compared to control. Cell proliferation on Ti-S and Ti-A-S was significantly greater than the rough surfaces for GCs (1.8 and 1.4 fold), while the differences were not significant for RCs.

B. Cell Differentiation

Alkaline Phosphatase Specific Activity: Cell layers of RCs cultured on both titanium rough surfaces, Ti-R and Ti-A-R, contained significantly less alkaline phosphatase specific activity (23% and 20%) than those from cultures grown on the plastic control (Figure 15). Conversely, GCs demonstrated a significant increase in cell layer alkaline phosphatase activity when grown on

Figure 13.

Number of resting zone chondrocytes (RC) and growth zone chondrocytes (GC) released by trypsinization from the different surfaces. RCs and GCs were cultured on plastic or Ti disks for 24 h after they had reached confluence on plastic. Data are from one of two replicate experiments, and the values shown are the mean \pm of six cultures. * $P < 0.05$, plastic vs. disk surfaces; ● $p < 0.05$ Ti-R vs. Ti-S; # $p < 0.05$ Ti-A-R vs. Ti-R.

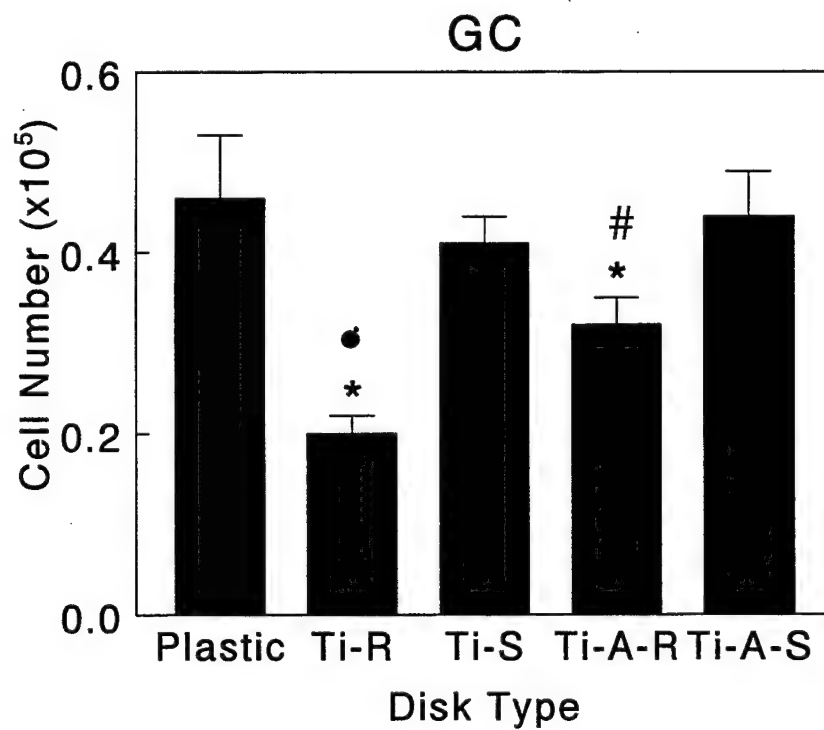
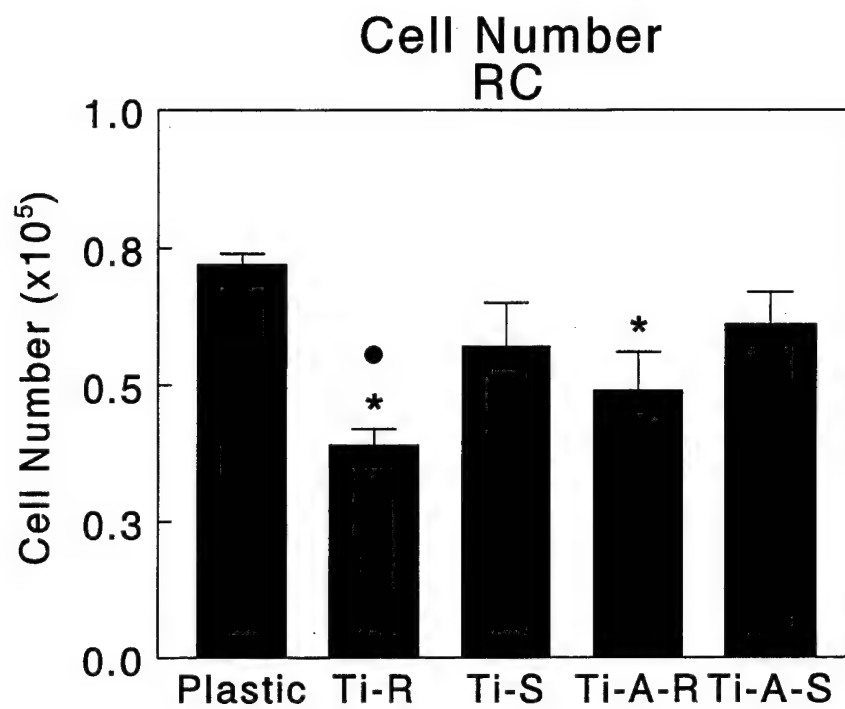
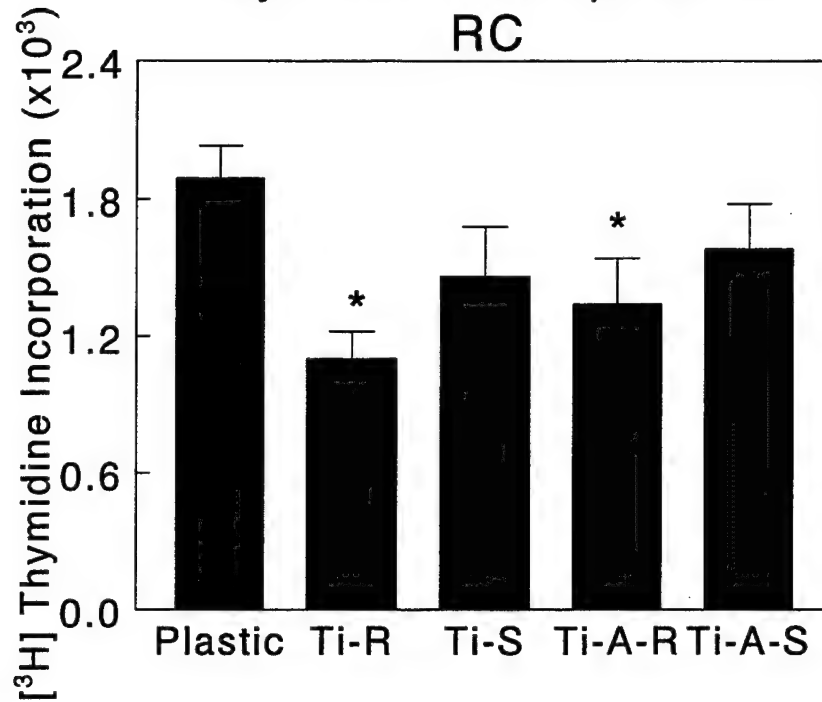


Figure 14.

[³H]-Thymidine incorporation by resting zone chondrocytes (RC) and by growth zone chondrocytes (GC) during culture on the different surfaces. After the cells had reached confluence on plastic, cells on all surfaces were made quiescent by reducing the serum concentration in the medium to 1% for 48 h. At that time, media were replaced with complete medium containing 1% FBS and [³H]-thymidine incorporation measured after 48 h. Data are from one of two replicate experiments, and the values shown are the mean \pm of six cultures. *P<0.05, plastic vs. disk surfaces; ●p<0.05Ti-R vs. Ti-S and Ti-A-R vs. Ti-A-S.

Thymidine Incorporation RC



GC

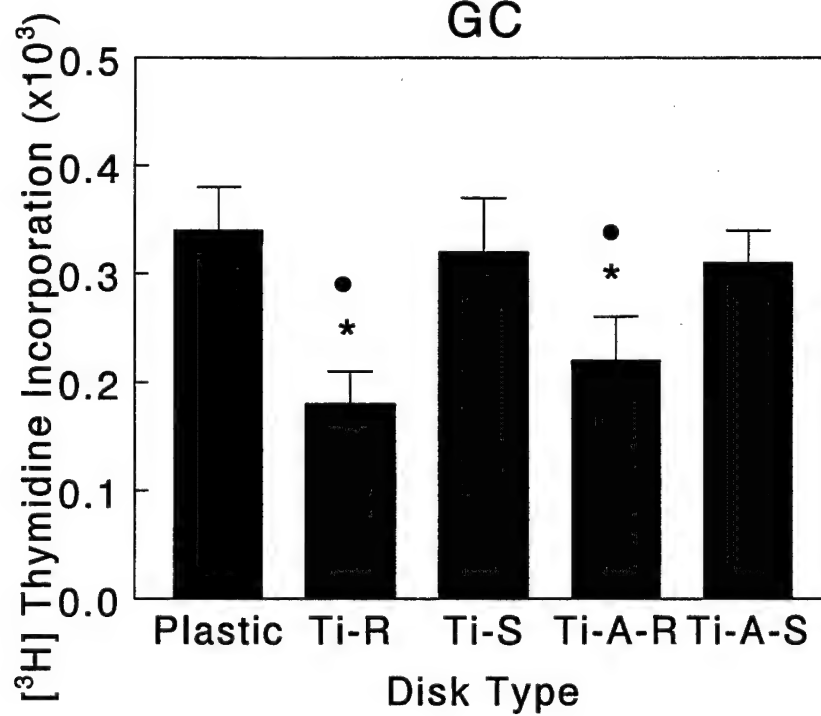
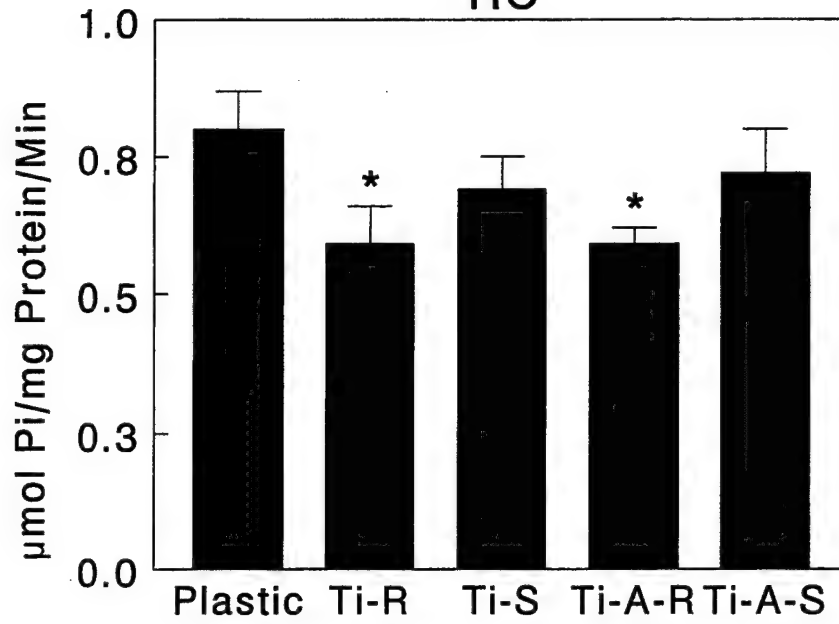


Figure 15.

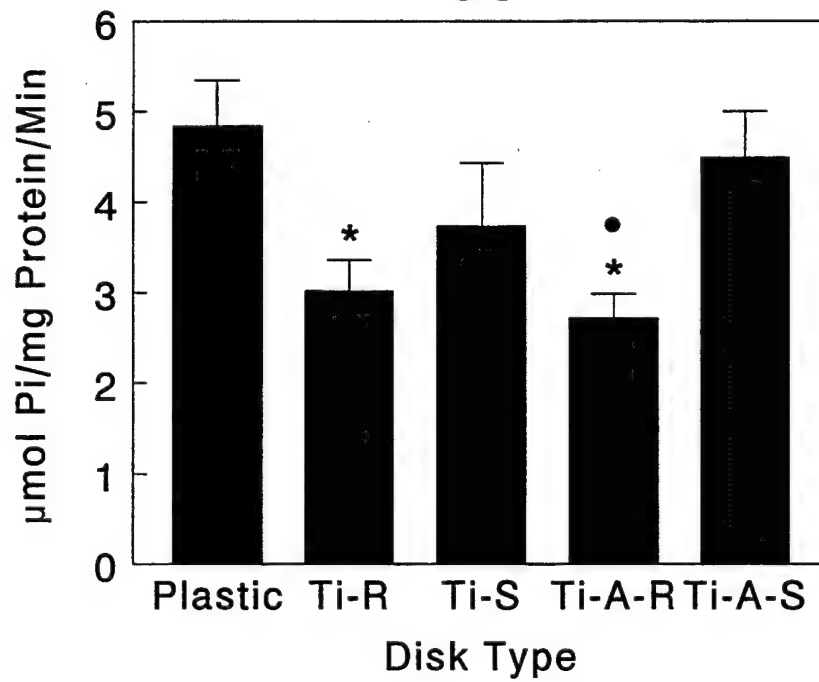
Alkaline phosphatase specific activity of resting zone chondrocytes (RC) and growth zone chondrocytes (GC) cultured on the different surfaces. RCs and GCs were cultured on plastic or Ti disks for 24 h after they had reached confluence on plastic, cultures were harvested by trypsinization and cells assayed for enzyme content. Data are from one of two replicate experiments, and the values shown are the mean \pm of six cultures. * $P < 0.05$, plastic vs. disk surfaces; ● $p < 0.05$ Ti-A-R vs. Ti-A-S.

Cell ALPase

RC



GC



Ti-R (1.2 fold) with respect to control and Ti-A-R disks, and 1.3 fold more enzyme specific activity than from Ti-S. Both RCs and GCs cultured on smooth surfaces, Ti-S and Ti-A-S, showed no difference in enzyme specific activity compared with control. However, in RCs there was a 19% decrease in enzyme specific activity for cultures on the Ti-A-R disks as compared to Ti-A-S disks.

When alkaline phosphatase specific activity in isolated RC and GC cells was measured, a surface roughness-dependent decrease was observed (Figure 16). Cell alkaline phosphatase specific activity was significantly decreased by 26% in RCs cultured on Ti-R and Ti-A-R, as well as GCs (38% and 43%) compared to the plastic control. Cells cultured on smooth surfaces showed no difference from control. Further, GCs cultured on Ti-A-S disks contained 1.6 fold more enzyme specific activity than those cultured on Ti-A-R.

C. Matrix Production

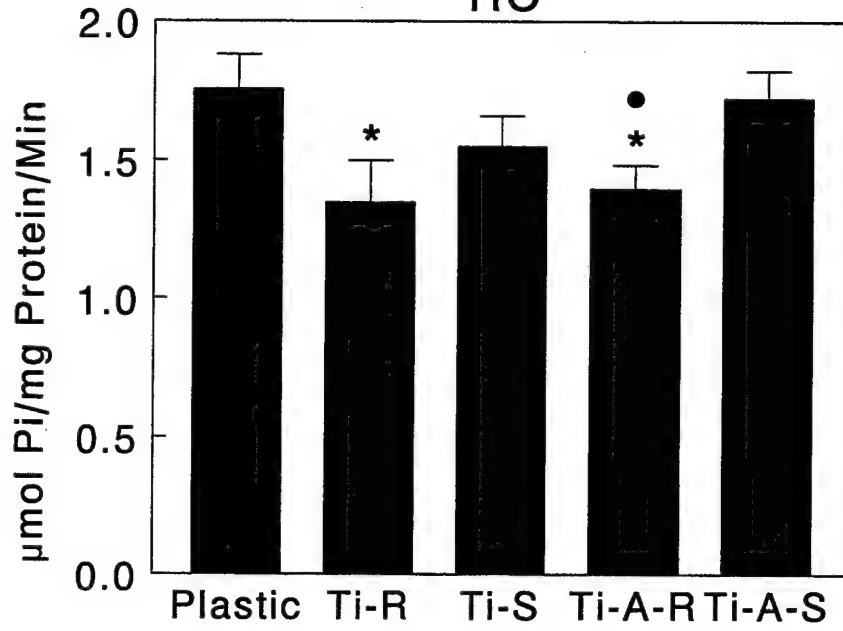
Collagen Synthesis: RCs cultured on Ti-R surfaces showed less percent collagen production than seen on all the other surfaces, but the differences were only significant when compared to cultures on the plastic control (Figure 17). RCs grown on Ti-R produced only 74% of the collagen of RCs cultured on the plastic control. In contrast, GCs showed a significant increase in percent collagen production when cultured on Ti-R surfaces (1.5 fold) compared with the to control. GCs cultured on Ti-R surfaces also produced 31% more collagen than GCs cultured on Ti-S surfaces. Further, GCs grown on Ti-R synthesized 1.2 fold more collagen than GCs from Ti-A-R. Although percent collagen production by GCs cultured on Ti-A-R disks appeared greater than control, the differences were not significant.

Proteoglycan Synthesis: Compared to control, [^{35}S] sulfate incorporation was significantly reduced on all metal surfaces by both RCs (47-68%) and GCs (29-46%) (Figure 18). The decrease in proteoglycan production and sulfation was greatest for RC and GC cells grown on Ti-R surfaces, demonstrating a possible chemical composition-dependent decrease as

Figure 16.

Alkaline phosphatase specific activity in the cell layers of resting zone chondrocytes (RC) and growth zone chondrocytes (GC) after culture on different surfaces. RCs and GCs were cultured on plastic or Ti disks for 24 h after they had reached confluence on plastic. Cultures were harvested by scraping the cell layer and the enzyme activity was determined. Data are from one of two replicate experiments, and the values shown are the mean \pm of six cultures. * $P < 0.05$, plastic vs. disk surfaces; ● $p < 0.05$ Ti-A-R vs. Ti-A-S.

Cell Layer ALPase RC



GC

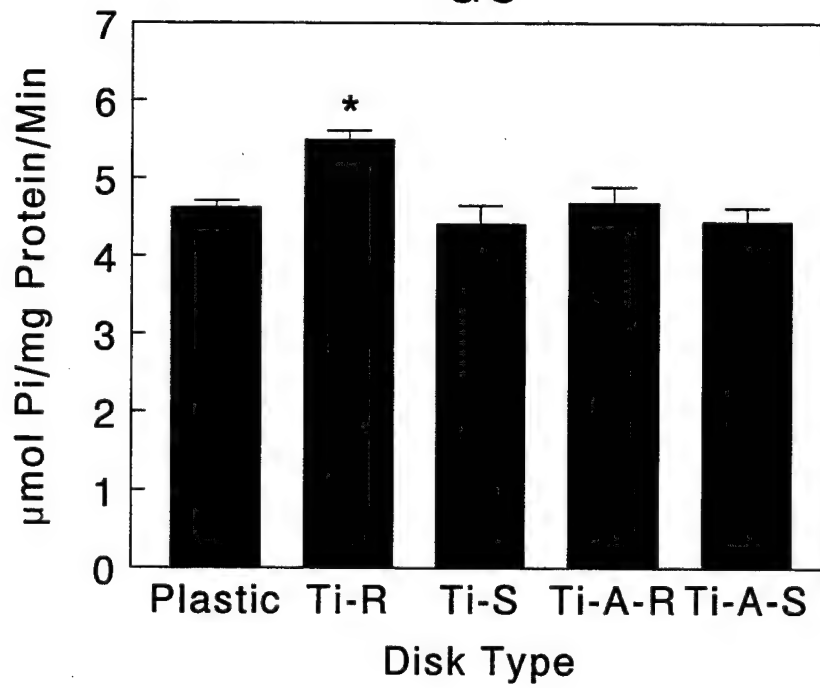


Figure 17.

Percent collagen production by resting zone chondrocytes (RC) and growth zone chondrocytes (GC) during culture on varying surfaces. RCs and GCs were cultured on plastic or Ti disks for 24 h after they had reached confluence on plastic. Data are from one of two replicate experiments, and the values shown are the mean \pm of six cultures.

* $P < 0.05$, plastic vs. disk surfaces; ● $p < 0.05$ Ti-R vs. Ti-S; # $p < 0.05$ Ti-A-R vs. Ti-R.

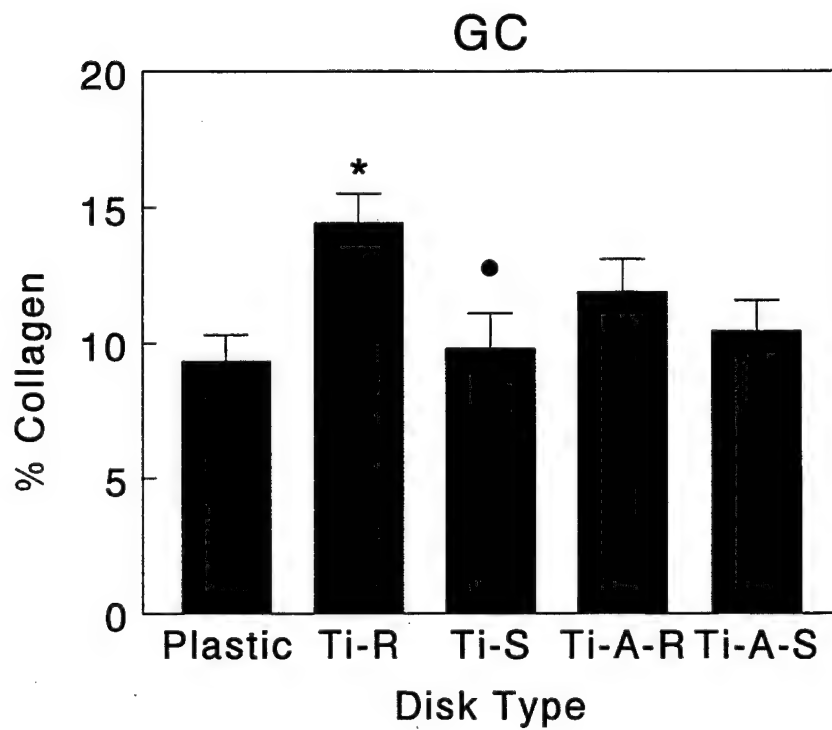
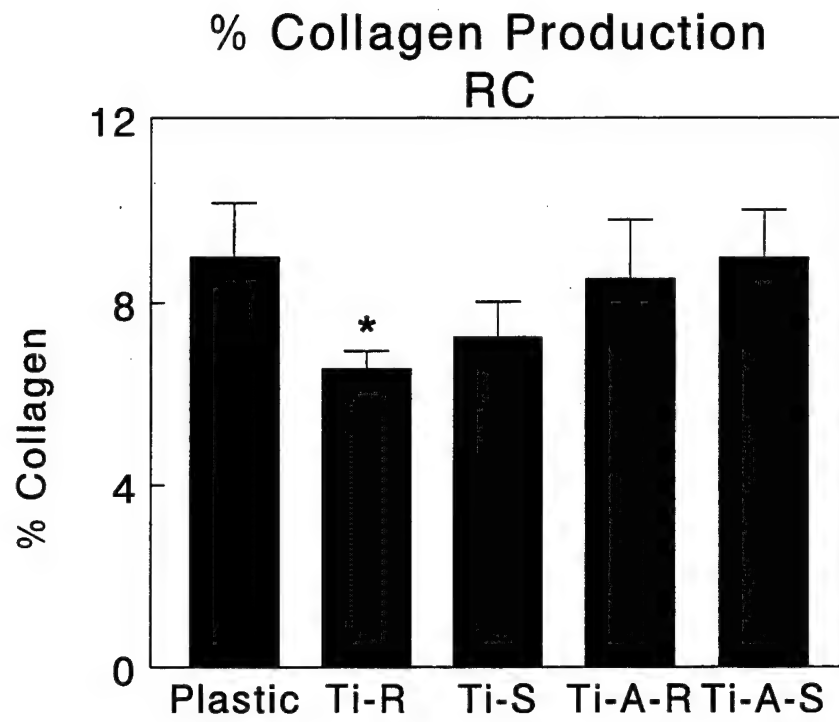
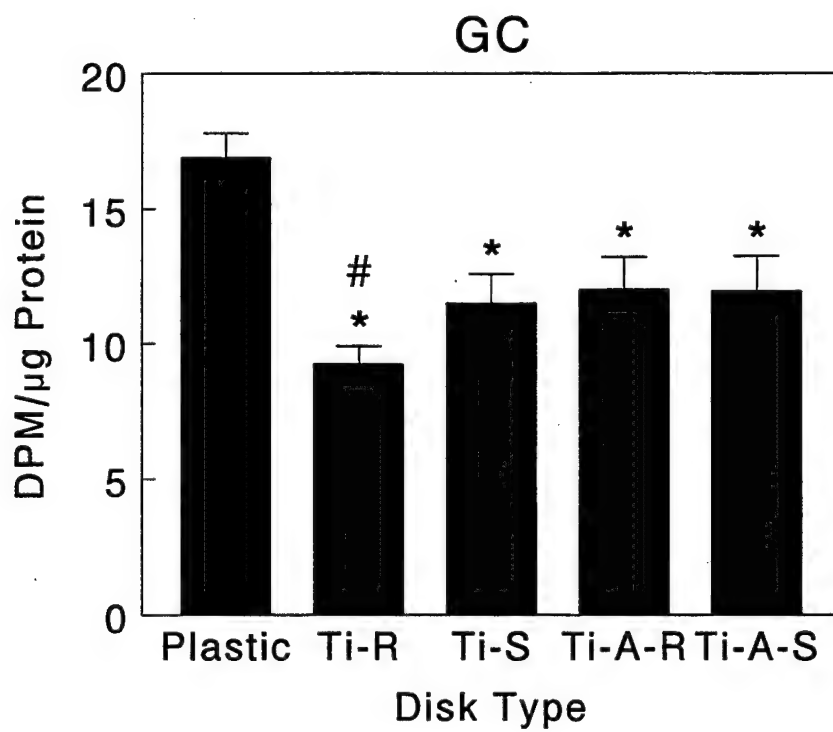
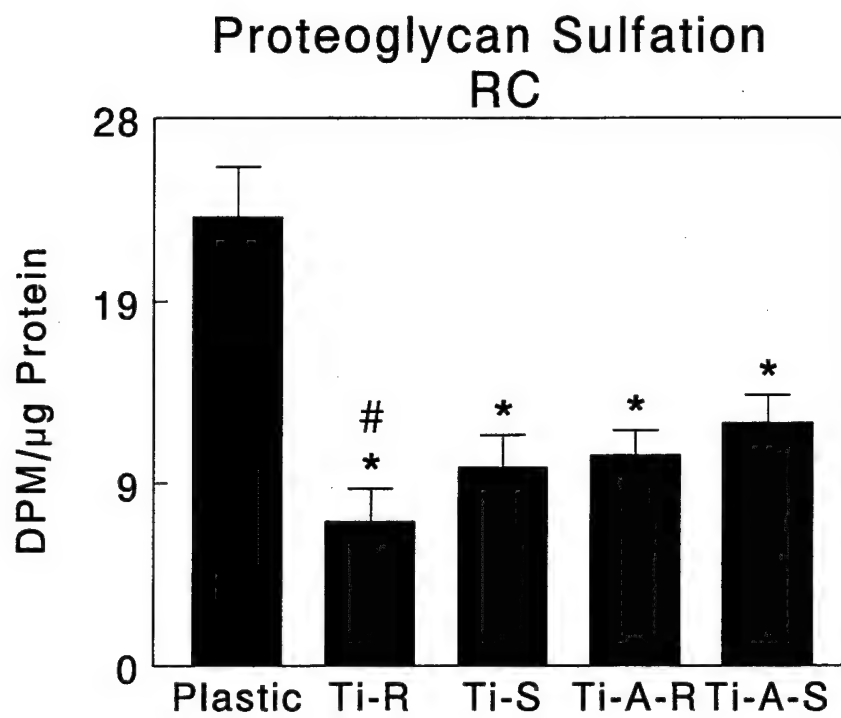


Figure 18.

[³⁵S]-Sulfate incorporation by resting zone chondrocytes (RC) and growth zone chondrocytes (GC) during culture on varying surfaces. RCs and GCs were cultured on plastic or Ti disks for 24 h after they had reached confluence on plastic. Data are from one of two replicate experiments, and the values shown are the mean \pm of six cultures.

*P<0.05, plastic vs. disk surfaces; #p<0.05 Ti-A-R vs. Ti-R.



compared to Ti-A-R. In RCs cultured on Ti-A-R a 1.5 greater [^{35}S] sulfate incorporation was observed compared to RCs from Ti-R. Similarly, in GCs cultured on Ti-A-R a 25% increase in [^{35}S] sulfate incorporation was observed compared to GCs from Ti-R.

D. Local Factor Production

TGF β Production: The production of TGF β was affected by surface roughness. TGF β production for RCs was significantly greater on both rough surfaces, Ti-R and Ti-A-R, than on smooth surfaces, Ti-S and Ti-A-S, and the plastic control (Figure 19). Ti-R produced 1.7 fold more TGF β than plastic control and Ti-S while Ti-A-R produced 1.3 fold more TGF β than control. In addition, RCs cultured on Ti-R disks produced 22% more TGF β than that seen on Ti-A-R cultures, which was statistically significant. GCs demonstrated significant differences for pure titanium rough surfaces as compared to control, Ti-S and Ti-A-R surfaces. GCs grown on Ti-R showed a significant increase in TGF β production (1.7 fold) compared to GCs from control. Likewise, GCs grown on Ti-R showed a 45% increase in TGF β production compared to GCs from Ti-S and Ti-A-R surfaces.

PGE $_2$ Production: PGE $_2$ production by both RCs and GCs was dependent on surface roughness (Figure 20). A significant increase in PGE $_2$ release was observed in RCs cultured on Ti-R (1.4 fold) and Ti-A-R (1.3 fold), and in GCs cultured on Ti-R (1.9 fold) and on Ti-A-R (1.5 fold) as compared to control. The amount of PGE $_2$ release on titanium smooth surfaces, Ti-S and Ti-A-S, was similar to control and significantly less than from rough surfaces for both cells. The PGE $_2$ release from RCs cultured on Ti-A-S was 22% less than that on Ti-A-R. GCs cultured on Ti-S produced 50% of the PGE $_2$ produced on Ti-R surfaces, while GCs grown on Ti-A-S produced 68% of the PGE $_2$ produced on Ti-A-R.

Figure 19.

Transforming growth factor β (TGF β) production by resting zone chondrocytes (RC) and growth zone chondrocytes (GC) during culture on varying surfaces. RCs and GCs were cultured on plastic or Ti disks for 24 h after they had reached confluence on plastic. At harvest, the media were collected, and TGF β content measured by ELISA. Values are the mean \pm SEM of six cultures. *P< 0.05, titanium vs. plastic; #P<0.05, Ti-A-R vs Ti-R; ●P<0.05, smooth vs. rough surface. Data are from one of two replicate experiments.

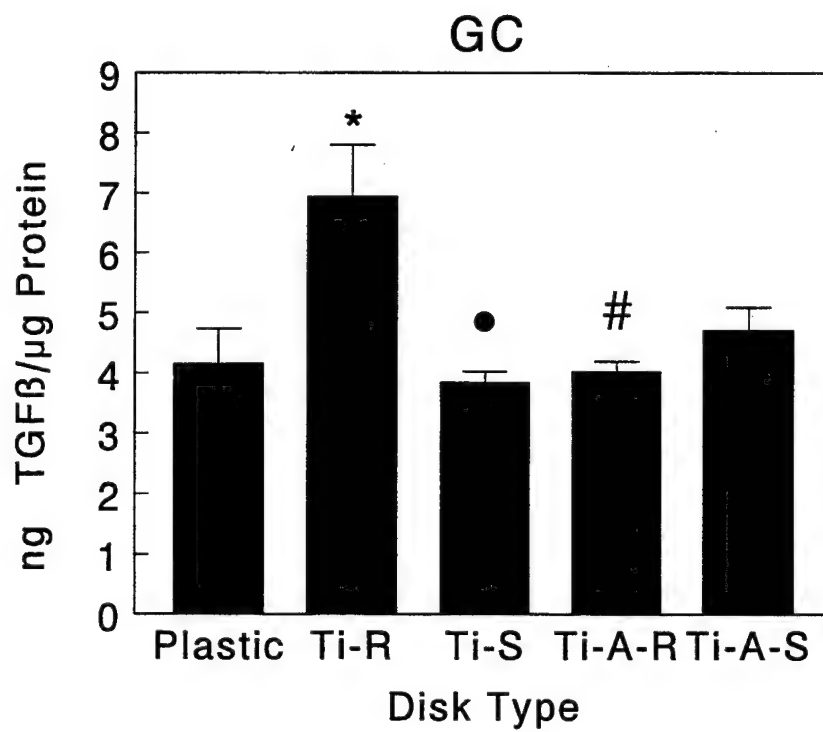
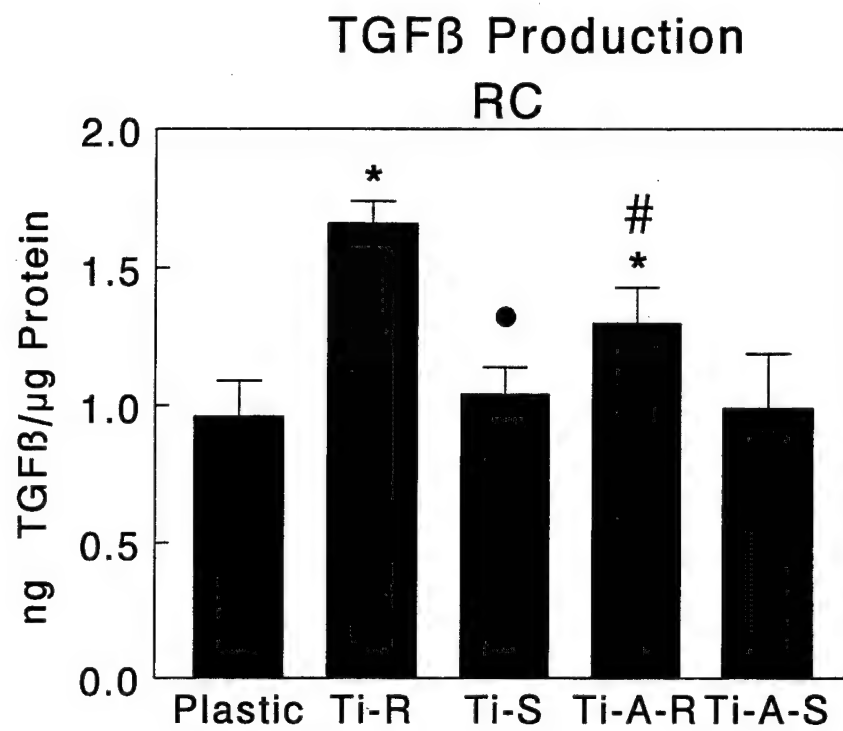
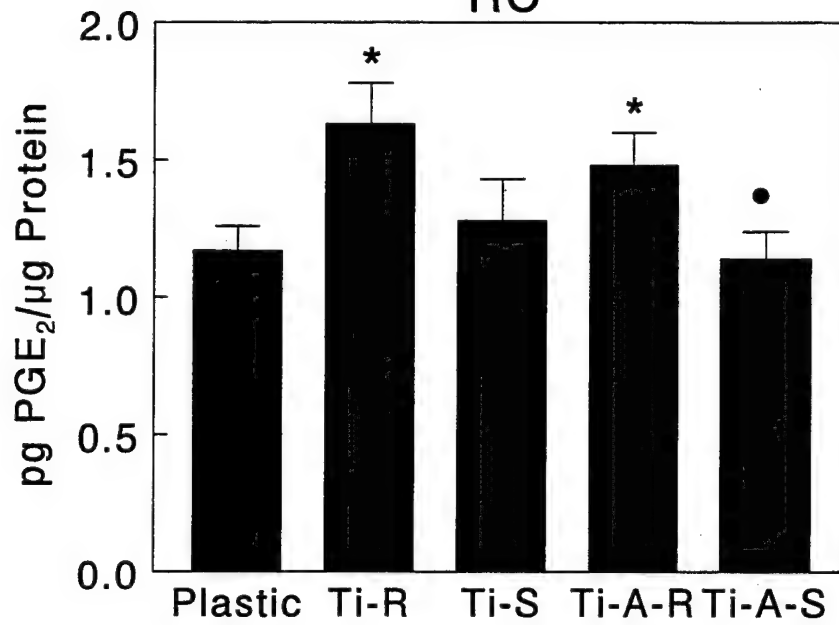


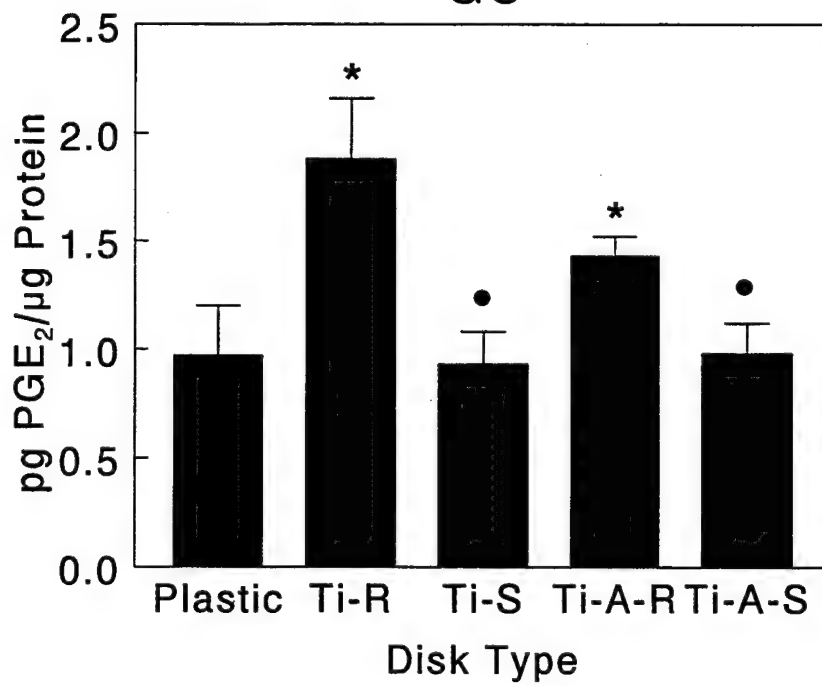
Figure 20.

Prostaglandin E₂ (PGE₂) production by resting zone chondrocytes (RC) and growth zone chondrocytes (GC) during culture on varying surfaces. RCs and GCs were cultured on plastic or Ti disks for 24 h after they had reached confluence on plastic. At harvest, the media were collected, and PGE₂ content measured by RIA. Values are the mean \pm SEM of six cultures. *P< 0.05, titanium vs. plastic; #P<0.05, Ti-A-R vs Ti-R; ●P<0.05, smooth vs rough surface. Data are from one of two replicate experiments.

PGE₂ Production RC



GC



IV. DISCUSSION

Previous studies have shown that bone cells are sensitive to differences in the surface characteristics of biomaterials (Schwartz et al., 1991, Hambleton et al., 1994, Martin et al., 1995, Thompson and Puleo, 1996, Boyan et al., 1998). It is evident from the present study that many aspects of cellular activity, including cell morphology, are affected by surface roughness and chemical composition in very specific ways. In particular, this study found that osteoblasts and chondrocytes demonstrate a more differentiated phenotype when cultured on pure titanium with a rough surface.

It has been hypothesized that the surface characteristics of biomaterials provide cells with a variety of signals and environmental cues that ultimately influence phenotypic expression (Boyan et al., 1996). In addition to affecting the ability of cells to adhere, certain biomaterial characteristics can affect the ability of cells to express integrins which regulate cellular morphology, matrix synthesis, and mineralization (Sinha and Tuan, 1996). Various "bone bonding" implant materials have been shown to regulate primary calcification of newly formed bone matrix by increasing matrix vesicle concentration and maturation (Schwartz et al., 1991). Another common theory is that rougher surfaces allow cells to migrate into pores and grooves forming stronger attachments (Wilke et al., 1990, Groessner-Schreiber and Tuan, 1992). The enhanced surface area of rough implants may lead to a greater amount of attachment proteins and regulatory factors binding to the surface heightening bone cell recruitment which may lead to greater osseointegration.

Controversy exists concerning the relative differences in cellular response to various methods of achieving rough surfaces or microtopography. In this study surface roughness was achieved by machine grinding the disks, resulting in a microtopography characterized by parallel grooves. Previous studies in our laboratory have used commercially pure titanium disks that have

been grit-blasted and acid-etched, producing a microtopography with random peaks and valleys (Martin et al., 1995, Schwartz et al., 1996, Boyan et al., 1998). When comparing the results using disks with equivalent R_a values but different microtopography similar effects on bone cell maturation and morphology were noted. In general, cells responded in a similar way to both plastic and smooth titanium surfaces. Similarly, the cells responded to both machined and grit blasted Ti surfaces of comparable R_a values in an almost identical fashion. Moreover, the morphology of MG63 cells cultured on the rough surfaces was more cuboidal in shape and similar to the morphology of cells cultured on grit-blasted surfaces. This morphology is typical of a more differentiated osteoblast (Groessner-Schreiber and Tuan, 1992). In contrast, MG63 cells on the smoother surfaces appeared flattened and dendritic or fibroblastic.

Cell morphology was also altered by chemical composition as shown by the differences in appearance of cells grown on Ti-R as compared to Ti-A-R. Cells cultured on Ti-R grew as a continuous monolayer and were rounded in appearance with cytoplasmic extensions spanning the grooves. In comparison, those on Ti-A-R appeared less cuboidal in shape and grew as a multilayer with an orientation parallel with the grooves. These findings suggest that the specific manufacturing technique to obtain a rough surface may not be as significant in determining cellular response as surface roughness as long as the R_a values can be sensed by the cells. The distance between peaks and not the manner in which roughness is achieved appears more significant. For all practical purposes, the distance between peaks should not exceed the ability of the cell to form focal attachments on two or more peaks, otherwise the cell would sense a rough surface as smooth. Furthermore, the results suggest that chemical composition can also alter bone cell morphology since the differences in roughness between the Ti-R and Ti-A-R surfaces was slight but the cell morphology was different.

As observed in similar studies by Martin et al. (1995) proliferation rate was negatively impacted by surface roughness. Both osteoblasts and chondrocytes cultured on surfaces of

increased roughness demonstrated decreased levels of [^3H]-thymidine incorporation and cell number as compared to smooth surfaces and plastic control. GCs were more sensitive than RCs to surface roughness showing greater decreases in cell number and [^3H]-thymidine incorporation between rough and smooth surfaces. In comparison, the chondrocytes showed no difference in proliferation when cultured on smooth Ti-A or Ti surfaces as compared to the plastic glow discharged tissue culture polystyrene control. The differential response between RCs and GCs suggests that surface roughness has a cell-maturation dependent influence on the chondrocytes. Previous studies have established that a reduction in cell proliferation is often correlated with increased differentiation (Lian and Stein, 1992, Rickard et al., 1993). In this model, this was confirmed by measuring other markers of bone cell differentiation such as ALPase activity for both chondrocytes and osteoblasts and osteocalcin for osteoblasts.

Rougher surfaces demonstrated increased ALPase, an early marker of enhanced differentiation, for MG63 cells and GCs, and decreased ALPase for RCs. While the differences between RCs and GCs concerning ALPase seems significant this response was expected since RCs are less mature and in a different state of maturation, typically demonstrating greater proliferation as opposed to differentiation. The production of this enzyme by RCs correlates with the response noted by the proliferation measures in which RCs showed a less dramatic reduction in cell number and [^3H]-thymidine incorporation than GCs. The heightened ALPase seen on rougher surfaces appeared to target the matrix vesicles since the increases in cell layer ALPase were significantly greater than those of isolated cell ALPase. In fact cell ALPase was significantly decreased in GCs compared with control, suggesting that chondrocytes modulate cellular ALPase in a manner distinct from matrix vesicle ALPase. While MG63 cells grown on smoother surfaces also exhibited decreased proliferation and had increased ALPase compared to control, the effects were less robust than those seen on rougher surfaces. Moreover, the findings that surface

roughness effects are targeted to the cell layer and therefore, the matrix vesicles, clearly suggest that surface roughness enhances bone cell differentiation and possibly calcification.

When osseointegration is the desired result, adequate and appropriate matrix production is necessary for the development of a calcifiable tissue. It is evident from this study and previous observations that surface roughness enhances matrix synthesis (Groessner-Schreiber and Tuan, 1992, Martin et al., 1995, Schwartz et al., 1996). Proteoglycan sulfation was reduced on all surfaces as compared to control with the greatest reductions noted on Ti-R for all cell types. As bone cells mature new production of proteoglycan is reduced as is sulfation of existing proteoglycan. In addition, proteoglycan aggregates must be degraded prior to calcification of the matrix (Dean et al., 1992, 1994), therefore a reduction in proteoglycan sulfation supports the hypothesis that the cells are more differentiated. However, it is impossible to determine whether the reduced proteoglycan sulfation found in this study is a result of osteoblast and chondrocyte maturation or slower production by less mature cells. Similarly, the synthesis of collagen on rougher surfaces was greater than on smoother surfaces for MG63 cells and GCs. The production of PGE₂ and TGFβ was greater on the rougher surfaces, supporting our previous observations that there is a positive correlation between these two factors and increasing surface roughness (Kieswetter et al., 1996). Further, results in this study were similar to those of Choi et al. (1996), in that increases in collagen production were correlated with increased expression of PGE₂ and TGFβ.

While the exact role of TGFβ and PGE₂ in calcification remains uncertain, the amounts of TGFβ produced on rough surfaces was comparable to those levels shown to stimulate osteoblast-like cells and chondrocytes (Bonewald et al., 1992, Schwartz et al., 1993, Kieswetter et al., 1996) while the levels of PGE₂ produced were within limits considered to be osteogenic and not inflammatory (Raisz and Fall, 1990). Surface roughness modulated the ability of MG63 cells, RCs and GCs to synthesize and secrete autocrine and paracrine mediators such as TGFβ and

PGE₂. Their release by the bone cells was markedly enhanced on rough surfaces while smooth surfaces produced levels that were essentially identical to the plastic control. Although a smaller number of cells were present on the rougher surfaces, as indicated by cell number, the total levels of TGFβ and PGE₂ were significantly greater. The combination of increased factor production and decreased proliferation on rough surfaces indicates that these cells synthesized more local factor on a per-cell basis. Taken together this indicates that bone cells grown on smooth surfaces failed to exhibit a more differentiated phenotype (Table 2). Furthermore, the increases were greatest on the Ti-R surfaces suggesting a chemical composition effect and supporting the contention that cells cultured on rough pure titanium surfaces exhibited a more differentiated phenotype.

While dramatic differences were noted in cellular response to rough and smooth surfaces, chemical composition also affected the maturation state of MG63 osteoblast-like cells and chondrocytes (Table 3). As shown by Auger electron spectroscopy, a titanium oxide layer formed on both the Ti and Ti-A surfaces, however differences in the surface chemistry did exist. In particular Al³⁺ ion was found on the surface of Ti-A, making it unlikely that the oxides were identical. This could have a direct effect on the conditioning film that forms as the material surface interacts with the culture medium and subsequently, alters cell adhesion, attachment and response (Jarcho et al., 1977, Norde 1992). The results of this study show that cells are sensitive to and can discriminate between minor differences in surface chemistries. Specifically, cells grown on Ti-R produced more ALPase, osteocalcin (MG63 cells), and local factors than those on Ti-A-R. Although [³H]-thymidine incorporation and cell number were reduced on all cultures, both MG63 cells and GCs had a significantly greater decrease on Ti-R when compared to Ti-A-R. This indicates that MG63 cells and GCs grown on Ti-A-R and Ti-R ceased to proliferate and initiated expression of the mature phenotype, however, cells on Ti-A-R did so at a slower rate. This hypothesis is supported by the fact that ALPase was elevated on Ti-A-R but to a lesser

Table 2.

Response of cells to rough vs. smooth surfaces.

| Assessment | MG63 | RCs | GCs |
|-----------------------------|------|-----|-----|
| Cell Number | ↓ | ↓ | ↓ |
| [³ H]-Thymidine | ↓ | -- | ↓ |
| Cell Layer ALPase | ↑ | ↓ | ↑ |
| Cell ALPase | ↑ | -- | ↓ |
| Osteocalcin | ↑ | n/a | n/a |
| Collagen Synthesis | ↑ | -- | ↑ |
| Proteoglycan Sulfation | -- | -- | -- |
| TGFβ Production | ↑ | ↑ | ↑ |
| PGE ₂ Production | ↑ | ↑ | ↑ |

Comparison of cell response to rough (Ti-R & Ti-A-R) and smooth (Ti-S & Ti-A-S) surfaces (↑ = R > S; ↓ = S > R; -- = no significant difference). MG63 osteoblast-like cells, RCs and GCs were cultured on the surfaces of the following types of disks: pure titanium, rough (Ti-R); titanium alloy, rough (Ti-A-R); pure titanium, smooth (Ti-S); titanium alloy, smooth (Ti-A-S). #p < 0.05, rough vs. smooth surface.

Table 3.

Response of cells to pure titanium vs. titanium alloy surfaces.

| Assessment | MG63 | RCs | GCs |
|-----------------------------|------|-----|-----|
| Cell Number | ↓ | -- | ↓ |
| [³ H]-Thymidine | -- | -- | -- |
| Cell Layer ALPase | ↑ | -- | ↑ |
| Cell ALPase | ↑ | -- | -- |
| Osteocalcin | ↑ | n/a | n/a |
| Collagen Synthesis | ↑ | -- | -- |
| Proteoglycan Sulfation | -- | ↓ | ↓ |
| TGFβ Production | ↑ | ↑ | ↑ |
| PGE ₂ Production | -- | -- | -- |

Comparison of cell response to pure titanium (Ti-R & Ti-S) and titanium alloy (Ti-A-R & Ti-A-S) surfaces (↑ = Ti > Ti-A; ↓ = Ti-A > Ti; -- = no significant difference). MG63 osteoblast-like cells, RCs and GCs were cultured on one of the following four disk surfaces: pure titanium, rough (Ti-R); titanium alloy, rough (Ti-A-R); pure titanium, smooth (Ti-S); titanium alloy, smooth (Ti-A-S). #p < 0.05, pure titanium vs. titanium alloy surface.

degree than seen on Ti-R and in MG63 experiments, cells cultured on Ti-A-R did not exhibit similar elevations in osteocalcin production. In fact, cells cultured on Ti-A-R produced only basal levels of osteocalcin, similar to smooth surfaces and control, indicating that chemical composition overrode the effects of roughness. Perhaps one possibility for these findings is that osteocalcin enhancement was inhibited in MG63 cells cultured on Ti-A-R surfaces. A previous report by Thompson and Puelo (1996) found suppression of rat bone marrow cell differentiation through inhibition of osteocalcin production when these cells were cultured on titanium alloy surfaces.

Collagen production by the bone cells was also modulated by surface composition. The production of collagen was decreased by 15% on Ti-A-R as compared to Ti-R for MG63 cells. Whereas, matrix synthesis by chondrocytes was modulated in a cell maturation specific manner with respect to chemical composition. Collagen production by RCs was significantly decreased on Ti-R surfaces while in GCs it was significantly increased on Ti-R as compared to control. In addition, local factor production was less on Ti-A-R as compared to Ti-R for all cell types. Taken together these findings suggest that pure titanium enhanced bone cell maturation as compared to titanium alloy. Secondly, osteogenesis might be further enhanced on the pure titanium material with a rough surface.

Despite the differences in cellular response to material composition, the results indicate that roughness remains the overriding variable in promoting osteogenic differentiation. The enhanced expression of a differentiated phenotype in response to surface roughness has been demonstrated by other studies using chick osteoblasts (Groessner-Schreiber and Tuan 1992), fetal rat bone marrow stromal cells (Stanford et al., 1994), chondrocytes (Hambleton et al, 1994, Schwartz et al., 1996), and MG63 cells (Martin et al., 1995, Kieswetter et al., 1996, Boyan et al., 1998). This study confirms previous observations that surface roughness can modulate the phenotypic expression of bone cells and is in agreement with the hypothesis of Lian and Stein (1992) regarding osteogenic cell differentiation. Furthermore, this study shows for the first time

that proliferation, differentiation, extracellular matrix synthesis, and local factor production in chondrocytes are affected by both surface roughness and the chemical composition of biomaterials. Additionally, the response of growth plate chondrocytes to titanium disks appears to depend on the state of endochondral maturation of the cells.

Future Studies

In this present study the surface roughness value for rough surfaces was 3.44 - 4.24 μm . Previous research in our laboratory has shown that cells respond favorably to R_a values around 4.0 μm . In these studies, cell proliferation was reduced but not blocked and phenotypic differentiation was enhanced. Future studies could use this information to determine optimal degree of surface roughness which enhances bone apposition eventually leading to modifications in implant manufacturing techniques. However, prior to alterations in implant manufacturing techniques and design, in vivo comparisons of implants with various microscopic surface characteristics are necessary to determine if the results of the in vitro experiments have in vivo relevance.

Summary

In order for osseointegration to occur a series of events which include protein absorption, cellular adherence, proliferation, differentiation, matrix synthesis, and calcification must occur at the bone-implant interface. Microscopic surface characteristics of implant materials can affect these various phases. While the exact surface characteristics that induce specific cell responses remain to be elucidated, this study suggests that surface roughness and chemical composition play a major role in the cellular response at the bone-implant interface. Further, these results suggest that a dental implant with a pure titanium surface and rough microtopography may better promote osteogenesis than one fabricated from Ti-alloy and/or has a smooth surface.

Optimal wound healing and apposition of new bone at the bone-implant interface are essential for the success and long-term function of dental and orthopaedic implants. This research has provided new insight into how surface roughness and chemical composition modulate bone cell behavior during the initial phases of healing. This information will hopefully aid in the development of an implant surface whose characteristics will enhance osteogenesis and maximize the potential for clinical success.

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VITA

Jack H. Lincks was born on July 13, 1961, to John Henry and Jeanne Marie Cerise Lincks in New Orleans, Louisiana. Following graduation from John Curtis Christian High School in 1979, he attended Loyola University of the South. He was selected for early entry into dental school in 1982, where Dr. Lincks received his Doctor of Dental Surgery degree from Louisiana State University School of Dentistry in 1986. As a junior and senior dental student Dr. Lincks was granted an NIH Pre-Doctoral Research Training Award and was selected to present his research in the Hatton competition at the American and International Association of Dental Research. His research was published in the Journal of Prosthodontics in 1989. During dental school he was elected to the C. Edmond Kells Honorary Society and upon graduation received the Pierre Fauchaud Award. Following graduation Dr. Lincks was commissioned as a Captain in the United States Air Force and since has been promoted to the rank of Lieutenant Colonel. Dr. Lincks has been stationed at Homestead AFB, FL, King Fahd AS, Saudi Arabia during the Persian Gulf War and Elmendorf AFB, AK as a General Dental Officer. Dr. Lincks received the Fellowship Award from the Academy of General Dentistry in 1994. He began periodontal residency training at Wilford Hall Medical Center, Lackland AFB, TX in 1996 and will graduate in June 1999. Dr. Lincks has been married to his wife Jennifer for 11 years and together they have a 5 year old daughter, Kaitlyn Cerise, a 3 year old son, Evan Michael, and a newborn baby boy, Brian Patrick. Upon completion of his training, he anticipates continuing his career and service with the United States Air Force, acquiring board certification in periodontics, teaching and practicing clinical periodontics.